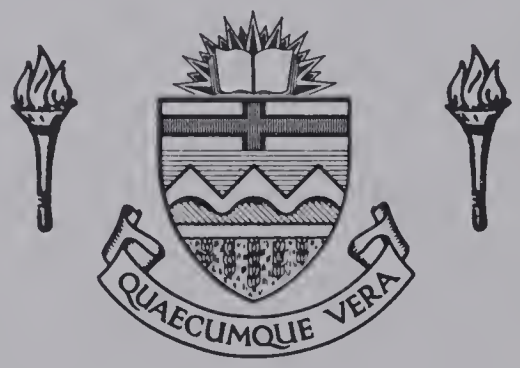
For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex libris
universitatis
albertaensis



THE UNIVERSITY OF ALBERTA

# REGULATION OF ONE-CARBON METABOLISM BY L-METHIONINE IN *SACCHAROMYCES CEREVISIAE*

by

© KIM-LOON LOR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

FALL, 1971

Thesis
1981 F
S58

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'Regulation of one-carbon metabolism by L-methionine in *Saccharomyces cerevisiae*' submitted by Kim-Loon Lor in partial fulfilment of the requirements for the degree of Master of Science.

# ABSTRACT

The levels of pteroylglutamates in cultures of *Saccharomyces cerevisiae* (ATCC 9763) grown in basal media with and without L-methionine, were examined during different phases of growth. The levels of these derivatives were markedly decreased by supplementing the media with L-methionine. A similar effect of this amino acid on the levels of conjugated pteroylglutamates was also observed.

Individual pteroylglutamate derivatives in the cells after 6 and 24 hours of growth were separated by DEAE-cellulose column chromatography. They were identified by co-chromatography with authentic samples, differential microbiological response with *Lactobacillus casei* (ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081) and by using γ-glutamyl carboxypeptidases from various sources. In extracts of cells grown in the presence of methionine, the levels of the methylated derivatives were drastically reduced. Smaller decreases in levels were also observed for formylated and unsubstituted derivatives when methionine was supplied.

Carboxypeptidase treatment of the extracts before chromatography resulted in large increases in the levels of formyl derivatives but did not appreciably change the levels of methylated derivatives. The formyl compounds which occurred as polyglutamyl derivatives were also greatly decreased by supplementing the cultures with L-methionine.

The effect of L-methionine on the levels and activities

of 10-formyltetrahydrofolate synthetase, 5,10-methylenetetrahydrofolate reductase, 5-methyltetrahydrofolate:homocysteine transmethylase and serine hydroxymethyltransferase were studied. Methionine was found to repress the transmethylase *in vivo* and inhibited the activity of the reductase *in vitro*. Serine hydroxymethyltransferase was not inhibited or repressed by L-methionine while 10-formyltetrahydrofolate synthetase activity was partially inhibited by this amino acid *in vitro*.

The effect of L-methionine on one-carbon metabolism was further examined *in vivo* by incubating the cells with formate-$^{14}C$ in the presence and absence of this amino acid. Incorporations of $^{14}C$ into various amino acids and S-adenosylmethionine were determined. Such feeding experiments indicated that methionine-grown cells, besides containing larger amounts of S-adenosylmethionine, had less capacity to convert formate-$^{14}C$ into methionine. However, these cultures readily incorporated formate-$^{14}C$ into serine and the adenosyl moiety of S-adenosylmethionine. In these experiments, labelled carbon was also incorporated into threonine and cystathionine, in each case the amounts of $^{14}C$ in these products was affected by the presence of exogenous methionine.

It is concluded that L-methionine plays an important role in regulating the biogenesis and utilization of methyl groups which arise from the pteroylglutamate pool. In addition, methionine also appears to regulate the biosynthesis of serine, adenine, threonine and cystathionine. The significance of these regulatory roles of methionine is discussed.

ACKNOWLEDGEMENTS

I wish to thank Dr. Edwin A. Cossins for assistance during this study and help in preparation of this thesis.

I wish to express thanks to Mrs. Betty Ford for typing this thesis.

The work contained in this thesis was supported by a grant from the National Research Council of Canada to Dr. E. A. Cossins.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

# LIST OF ABBREVIATIONS

The abbreviations used are those commonly found in biochemical literature. Pteroylglutamate derivatives are designated by the abbreviations suggested by the IUPAC-IUB Commission as listed in *The Biochemical Journal* 102 (1967) 15, as follows:

| | |
|---|---|
| PteGlu | : Pteroylglutamic acid |
| $H_2$PteGlu | : Dihydropteroylglutamic acid |
| $H_4$PteGlu | : Tetrahydropteroylglutamic acid |
| 5-HCO-$H_4$PteGlu | : 5-formyltetrahydropteroylglutamic acid |
| 5-$CH_3$-$H_4$PteGlu | : 5-methyltetrahydropteroylglutamic acid |
| 10-HCO-$H_4$PteGlu | : 10-formyltetrahydropteroylglutamic acid |
| 5,10-$CH_2$=$H_4$PteGlu | : 5,10-methylenetetrahydropteroylglutamic acid |
| 5,10-CH≡$H_4$PteGlu | : 5,10-methenyltetrahydropteroylglutamic acid |
| 5-HCN-$H_4$PteGlu | : 5-formiminotetrahydropteroylglutamic acid |
| $PteGlu_2$ | : Pteroyldiglutamic acid |
| $PteGlu_3$ | : Pteroyltriglutamic acid |
| SAM | : S-adenosyl-L-methionine |
| SAHC | : S-adenosyl-L-homocysteine |
| DEAE-cellulose | : Diethylaminoethyl cellulose |

# INTRODUCTION

The growth and maintenance of cells requires a highly integrated interplay of catabolism and anabolism. The regulation of this metabolic machinery is of primary importance to the economy of the cell.

The biosynthesis of methionine, an important amino acid in protein synthesis and also a major source of methyl groups, is now known to involve the pteroylglutamate metabolism (Blakley, 1969). Increased knowledge of methionine biosynthesis, transmethylation reactions and the important role of pteroylglutamates has led to an examination of the regulation of these integrated metabolic pathways.

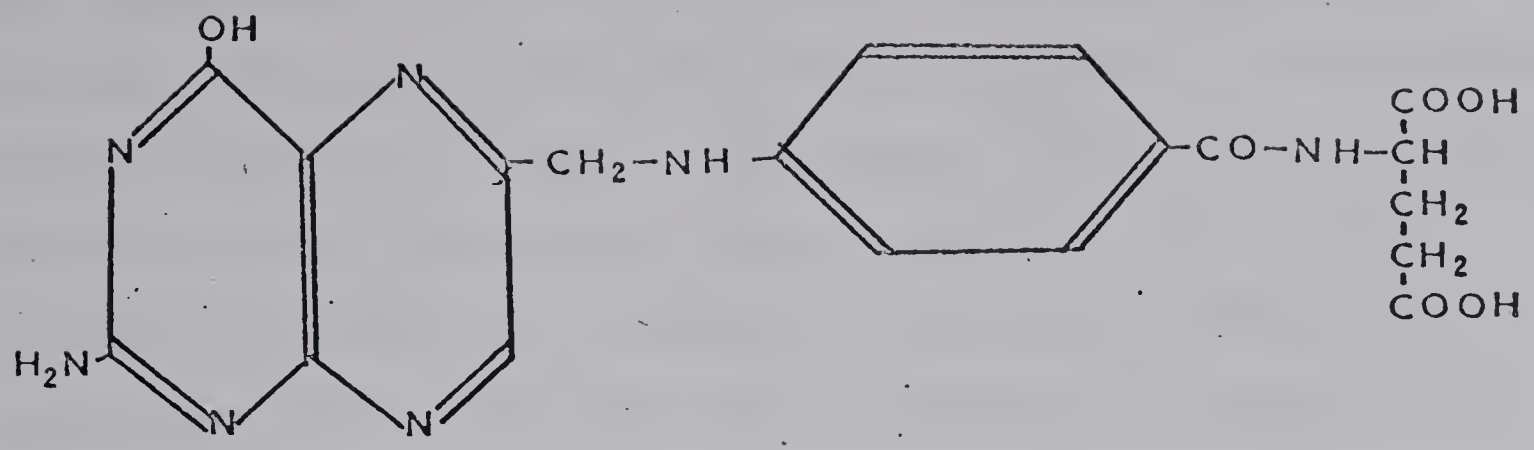
In general, metabolic control at the cellular level is achieved by two distinct types of mechanisms. One involves repression of protein synthesis, in which, the product of the reaction sequence serves as a repressor and inhibits the biosynthesis of the enzyme which catalyzes the reaction involved in the sequence. Jacob and Monod (1961) proposed the operon model for this regulatory system. In each operon model, there is a regulatory gene which specifies the structure of a repressor. The repressor binds to the operator gene and thereby inhibits the transcription of the structural gene of that operon. Repressors become active only in the presence of a corepressor which are generally of small molecular size. Repressors have since been shown to be proteins (Gilbert and Müller-Hill, 1966; Riggs and

Bourgeois, 1968). The action of the repressor can, however, be cancelled by the presence of an inducer. In the presence of an inducer, the repressor undergoes conformational changes and no longer binds to the operator gene and thus normal synthesis of that protein can occur (Gilbert and Müller-Hill, 1967). Repression is primarily concerned with an economy of protein synthesis.

The second type of control mechanism is product inhibition. In this control mechanism, a product of the reaction sequence serves, at physiological concentration, as a noncompetitive inhibitor of an enzyme which catalyzes a reaction involved in the sequence. Inhibitory action of this type is mainly due to the allosteric nature of end-product sensitive enzymes (Monod and Jacob, 1961). This type of regulation has been amply demonstrated for many biosynthetic pathways operating in microorganisms. In this respect it should, however, be noted that as the details of pteroylglutamate metabolism and methionine biosynthesis have only been recently elucidated, relatively little information on the regulation of pteroylglutamate-mediated metabolism is available.

*Discovery and Occurrence of Pteroylglutamate Derivatives*

The discovery of pteroylglutamates followed the studies of nutritional factors in yeast and liver extracts (Wills, 1931; Hogan and Stewart, 1935). PteGlu has the following structural formula (Angies *et al.*, 1946):




The alternative name "folic acid" was first proposed by Mitchell *et al.*, in 1941, for the substance they isolated from spinach leaves and which incidentally was also a nutritional factor for *Streptococcus faecalis* R. Derivatives of PteGlu act as coenzymes in many metabolic reactions (Blakley, 1969). These coenzymes are primarily concerned with the transfer of one-carbon units at the oxidation levels of formate, formaldehyde and methanol and in transforming these C-1 units from one oxidation state to another. The binding sites for C-1 units are known to be the N-5 and N-10 positions. These derivatives may be present as such or in conjugated forms with various numbers of glutamic acid residues attached in γ-peptide linkage.

Pteroylglutamates are commonly assayed microbiologically using *Lactobacillus casei*, *Streptococcus faecalis* R and *Pediococcus cerevisiae* (Bakerman, 1961). In such assays of pteroylglutamate content, the assay media contains all of the factors known to support growth of the bacteria with the exception of pteroylglutamate. Growth obtained on addition of tissue extracts is taken as a measure of pteroylglutamate content. However, variation in microbiological response to

the various derivatives is known to occur, the basis for this includes the type of derivative and the degree of conjugation (Wittenberg *et al.*, 1962). For example, these workers found that the formyl derivatives with 2 glutamic acid residues are approximately 50% as active as the corresponding monoglutamates for *S. faecalis* and *P. cerevisiae*. However, all diglutamyl derivatives are as active as the respective monoglutamates for *L. casei* (Noronha and Silverman, 1962). Generally, the method of choice for measurement of total "folic acid" content employs *L. casei* as the test organism (Eigen and Shockman, 1963). Differences in the specificity of the growth response of *L. casei*, *S. faecalis* and *P. cerevisiae* can then be used as a basis for the identification of naturally occurring pteroylglutamate derivatives. To determine derivatives in which more than two glutamic acid residues are linked to PteGlu, it is necessary to employ treatment with γ-glutamyl carboxypeptidases from chicken pancreas (Mims and Laskowski, 1945) hog kidney (Bird *et al.*, 1946) or from pea cotyledons (Roos, 1971) to hydrolyze the derivatives to forms capable of supporting the growth of the assay bacteria. Many biological materials contain such an enzyme (Bird *et al.*, 1945; Mims *et al.*, 1944; Laskowski *et al.*, 1945; Simpson and Schweigert, 1949) but such activity is apparently lacking in yeast (Volcani and Margalith, 1957).

Substantial improvement in the methods of pteroylglutamate isolation and DEAE-cellulose chromatography has led to better resolution of these compounds than was

possible in the earlier techniques of paper chromatography and bioautography (Winsten and Eigen, 1950; Wieland *et al.*, 1952). In DEAE-cellulose chromatography, the derivatives are eluted from the exchanger by a gradient of phosphate buffer in the presence of ascorbate (Silverman *et al.*, 1961; Sotobayashi *et al.*, 1966). The inclusion of ascorbate is necessary for protection of derivatives subject to oxidation.

Following these improvements in techniques for assay and isolation of pteroylglutamate derivatives, naturally occurring derivatives have been examined in several species. In Baker's yeast (*Saccharomyces cerevisiae*), more than 98% of the derivatives are conjugated with more than three glutamate residues (Schertel *et al.*, 1965). The major derivative appeared to be 5-$CH_3$-$H_4$PteGlu. In addition, 10-HCO-$H_4$PteGlu, 5-HCO-$H_4$PteGlu, $H_4$PteGlu and their dihydro derivatives were also present (Schertel *et al.*, 1965). However, no positive identification of these compounds, or their polyglutamate derivatives, was carried out.

The occurrence of pteroylglutamate derivatives has also been demonstrated in various microorganisms (Allfrey and King, 1950; Wright, 1955, 1956; Hakala and Welch, 1957). In most animal tissues, the principle derivative present is 5-$CH_3$-$H_4$PteGlu (Cropper and Scott, 1966; Noronha and Aboobaker, 1963) with lesser amounts of 5- and 10-HCO-$H_4$PteGlu present mainly as polyglutamates. In plants, 5-$CH_3$-$H_4$PteGlu has been found to be the major pteroylglutamate in pea cotyledons (Roos *et al.*, 1968) and in other plants

(Rohringer *et al.*, 1969; Shah *et al.*, 1970). Apart from 5-$CH_3$-$H_4$PteGlu, 5-HCO-$H_4$PteGlu and 10-HCO-$H_4$PteGlu are also predominant in the pteroylglutamate pool of plants and occur to some extent as conjugated derivatives (Iwai *et al.*, 1959).

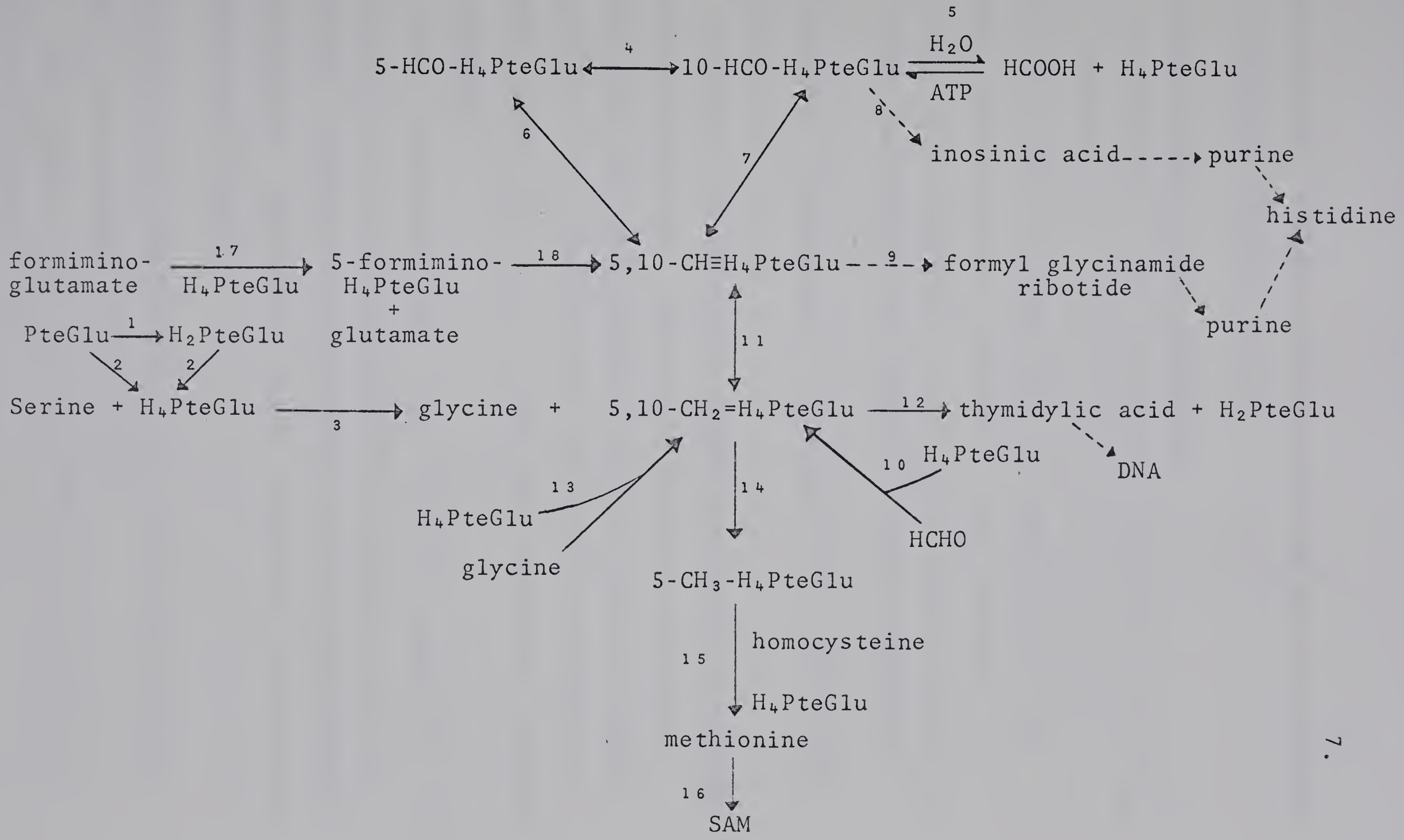
*Interconversion and Metabolic Function of Various PteGlu Derivatives*

The key reactions involved in the generation and interconversion of pteroylglutamate derivatives is summarized in Scheme 1. $H_4$PteGlu, which is formed from enzymic reduction of PteGlu or $H_2$PteGlu (reaction 2), is the major pteroylglutamate derivative capable of accepting C-1 units. $H_2$PteGlu can be formed from PteGlu (reaction 1) although the physiological significance of this reaction is not clear as $H_2$PteGlu can also be readily formed from other pteridines (Iwai *et al.*, 1969). The carbon-one unit can be derived from formate (reaction 5), serine (reaction 3), formaldehyde (reaction 10), the formimino group of formiminoglutamic acid (reaction 17, 18) and glycine (reaction 13). The methyl groups of choline and methionine (Siekevitz and Greenberg, 1950), acetone (Sakami, 1950), dimethylglycine and sarcosine (MacKenzie, 1950; Mackenzie and Abeles, 1956) have also been shown to be the source of carbon-one units for biological syntheses. The one-carbon unit of these pteroylglutamate derivatives can be oxidized and reduced enzymatically (Scheme 1) and as a result C-1 units can be utilized in the synthesis of purines (reactions 8 and 9), pyrimidines

SCHEME 1.

| REACTION NUMBER | TRIVIAL NAME | SYSTEMATIC NAME | E.C. NO. |
|---|---|---|---|
| 1 | Dihydrofolate dehydrogenase | 7,8-dihydrofolate:NADP oxidoreductase | 1.5.1.4 |
| 2 | Tetrahydrofolate dehydrogenase | 5,6,7,8-tetrahydrofolate:NADP oxido-reductase | 1.5.1.3 |
| 3 | Serine hydroxymethyltransferase | L-serine:tetrahydrofolate 5,10-hydroxy-methyltransferase | 2.1.2.1 |
| 4 | Formyltetrahydrofolate mutase | | |
| 5 | Formyltetrahydrofolate synthetase | formate:tetrahydrofolate ligase (ADP) | 6.3.4.3 |
| 6 | 5-formyltetrahydrofolate cyclodehydrase | | |
| 7 | Methenyltetrahydrofolate cyclohydrolase | 5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing) | 3.5.4.9 |
| 8 | Phosphoribosyl-amino-imidazole-carboxamide formyltransferase | | |
| 9 | Phosphoribosyl-glycineamide formyltransferase | | |
| 10 | Non-enzymic reaction | | |
| 11 | 5,10-methylenetetrahydrofolate dehydrogenase | 5,10-methylenetetrahydrofolate:NADP oxidoreductase | 1.5.1.5 |
| 12 | Thymidylate synthetase | | |
| 13 | Glycine "splitting enzyme" | | |
| 14 | 5,10-methylenetetrahydrofolate reductase | 5-methylenetetrahydrofolate:NAD oxido-reductase | 1.1.1.68 |
| 15 | 5-methyltetrahydrofolate: homocysteine methyltransferase | | |
| 16 | S-adenosylmethionine synthetase | | |
| 17 | Glutamate formimino transferase | N-formimino-L-glutamate:tetrahydrofolate 5-formiminotransferase | 2.1.2.5 |
| 18 | Formiminotetrahydrofolate cyclodeaminase | 5-formiminotetrahydrofolate ammonia-lyase (cyclizing) | 4.3.1.4 |

SCHEME 1. INTERCONVERSION AND THE METABOLIC FUNCTION OF THE VARIOUS PTEGLU DERIVATIVES



(reaction 12), histidine, proteins and the *de novo* synthesis of methionine (reaction 14, 15).

*Regulation of One-carbon Metabolism*

In contrast to the considerable information concerning the interconversion of pteroylglutamate derivatives, relatively little is known about the regulation of the enzymes catalyzing these reactions. The following is a review of basic properties of the major enzymes involved in one-carbon metabolism with emphasis on their regulation.

*Dihydrofolate dehydrogenase* (7,8-dihydrofolate:NADP oxidoreductase E.C. 1.5.1.4)

PteGlu is enzymatically reduced to $H_2$PteGlu by dihydrofolate dehydrogenase. This enzyme has been examined in *Clostridium sticklandii* (Bertino *et al.*, 1963), is NADPH dependent and is coupled to the conversion of pyruvate to carbon dioxide and acetyl CoA with the concomitant formation of $H_2$PteGlu:

$$\text{PteGlu} + \text{pyruvate} + \text{CoA} \longrightarrow H_2\text{PteGlu} + \text{acetyl CoA} + CO_2.$$

The enzyme is not inhibited by PteGlu antagonists and is, therefore, different in this respect from tetrahydrofolate dehydrogenase. Regulation of this enzyme has not yet been demonstrated to date.

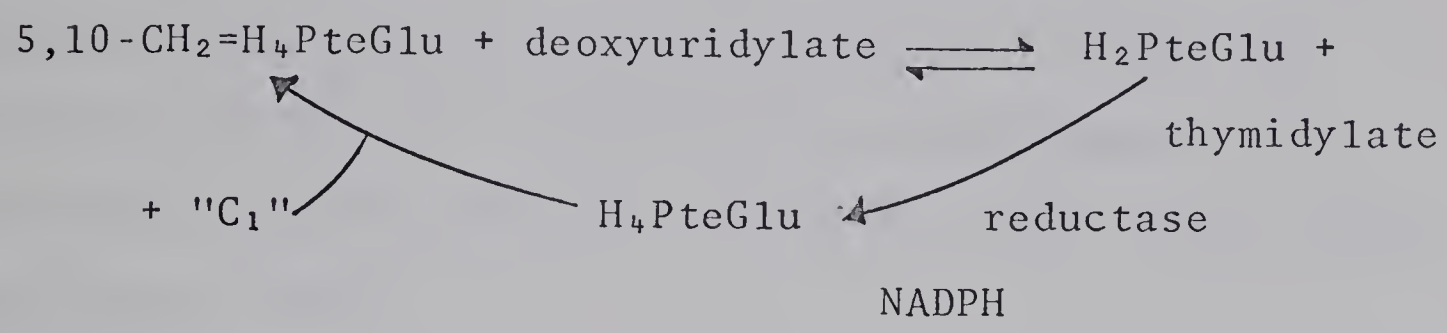
*Tetrahydrofolate dehydrogenase* (5,6,7,8-tetrahydrofolate: NAD(P) oxidoreductase (E.C. 1.5.1.3).

This enzyme is not only involved in the formation of $H_4$PteGlu from PteGlu, *e.g.*

$$H_2PteGlu + NADPH + H^+ \longrightarrow H_4PteGlu + NADP^+$$

$$PteGlu + 2NADPH + 2H^+ \longrightarrow H_4PteGlu + 2NADP^+$$

But is also closely associated with thymidylate biosynthesis:




In this latter capacity, this enzyme serves to reduce the $H_2PteGlu$ formed in the formation of thymidylate, thus regenerating $H_4PteGlu$. This enzyme has been extensively purified from bacteria (Hillcoat and Blakley, 1966; Burchall and Hitchings, 1965), mouse tumors (Bertino *et al.*, 1965; Zakrzewski *et al.*, 1966), calf thymus (Greenberg *et al.*, 1966) and chicken liver (Mell *et al.*, 1966; Kaufman and Gardiner, 1966). Surveys of the levels of tetrahydrofolate dehydrogenase activity in tissues of various mammalian tissues have been reported (Bertino *et al.*, 1964; Roberts and Hall, 1963; Braganca and Kenkare, 1964). The critical role of this enzyme in the formation of $H_4PteGlu$, and consequently of all pteroylglutamate coenzymes, indicates that a mechanism to control its synthesis would be of importance to the cell. Albrecht and Hutchison (1964) have shown that adenine does not repress the formation of the reductase in growing cultures of *S. faecalis*, but Burchall and Hitchings (1965) have shown that in the wild and trimethoprim-resistant strains of *E. coli*, grown in minimal medium in the presence

of adenosine, guanosine and thymidine, the specific activity of the reductase was decreased. Further decreases in specific activity were observed when these three end-products were present. In *L. casei*, when the concentration of PteGlu present in the growth medium exceeded $1.4 \times 10^{-9}$M, the specific activity of tetrahydrofolate dehydrogenase was decreased, possibly through repression of the enzyme (Ohara and Silber, 1969).

*5,10-Methylenetetrahydrofolate dehydrogenase* (5,10-methylene-tetrahydrofolate:NADP oxidoreductase E.C. 1.5.1.5).

This enzyme catalyzes the reaction:

$$5,10\text{-}CH{\equiv}H_4PteGlu + NADPH + H^+ \longleftrightarrow 5,10\text{-}CH_2{=}H_4PteGlu + NADP^+$$

The enzyme was first discovered by Jaenicke (1956) in pig and pigeon liver preparation. The requirements and stoichiometry of the dehydrogenase reaction was subsequently described by Uyeda and Rabinowitz (1967). Highly purified preparations of the enzyme have been obtained from yeast (Ramasastri and Blakley, 1962), *E. coli* (Donaldson *et al.*, 1965), calf thymus (Yeh and Greenberg, 1965), *Salmonella typhimurium* (Dalal and Gots, 1967; Ramasastri and Blakley, 1964) and in higher plants (Cossins *et al.*, 1970). The position of this enzyme within the pteroylglutamate pathway is of particular importance. The equilibrium constant of the reaction favors the formation of $5,10\text{-}CH_2{=}H_4PteGlu$. $5,10\text{-}Ch_2{=}H_4PteGlu$ is required for the formation of purine and also takes part in the interconversion of serine and glycine

(Wright, 1955; Cossins and Sinha, 1966). It has been shown in an amethopterin-resistant strain of *S. faecalis* grown under conditions that allowed for only limited serine biosynthesis, that derepression of the enzyme occurred to a level three times that found in cells grown in a medium containing casein hydrolysate as a source of serine. Clearly, the synthesis of this enzyme is controlled by the level of serine in the medium. Repression of this enzyme by serine in an amethopterin-resistant strain of *S. faecalis* was further confirmed by Albrecht (1966). Purines had no significant effect on this enzyme activity *in vitro* (Albrecht *et al.*, 1966). Exogenous additions of inosine or guanosine was found to cause a 35-45% decrease in the specific activity of the dehydrogenase in *E. coli* K12 (Taylor *et al.*, 1966). In contrast, Dalal and Gots (1966) have found that 5,10-$CH_2$=$H_4$PteGlu dehydrogenase activity was not repressed when *Salmonella typhimurium* LT-2 was grown in the presence of adenine (50 µg/ml). Inhibitors of purine biosynthesis, such as psicofuranine and 6,diazo-5,oxo-L-norleucine, failed to depress the levels of the dehydrogenase. However, when the size of the endogenous purine pool was decreased by growing a purine auxotroph of *S. typhimurium*, pur E-11, on limiting concentrations of xanthine, the specific activity of the enzyme rose 2-3 fold. Concentrations of PteGlu in the culture media did not alter the activity of this enzyme in *Lactobacillus casei* (Ohara and Silber, 1969). In *S. cerevisiae*, mutation at the ad3 locus caused a simultaneous

requirement for adenine and histidine (Roman, 1956). Jones and Magasanik (1967) reported that ad3 mutations result in 10-15% of the wild type activity for methylenetetrahydrofolate dehydrogenase. This explains in a satisfactory way the adenine requirement in ad3 mutants. Similar results have been obtained in *Schizosaccharomyces pombe* (Nagy *et al.*, 1969). Lazowska and Luzzati (1970a) have shown that in ad3 mutants the dehydrogenase activity is 50% of the activity in the wild type under appropriate assay conditions. Further studies by these workers (Lazowska and Luzzati, 1970b) have shown that there are two forms of methylenetetrahydrofolate dehydrogenase present in extracts of the wild type, one of which is apparently absent in ad3 mutants.

*5,10-methylenetetrahydrofolate reductase* (5-methylenetetrahydrofolate:NAD oxidoreductase E.C. 1.1.1.68)

This enzyme catalyzes the reaction:

$$5,10\text{-}CH_2{=}H_4PteGlu + FADH_2 \longrightarrow 5\text{-}CH_3\text{-}H_4PteGlu + FAD$$

This enzyme is important in the *de novo* formation of methyl groups from pteroylglutamate pools. $5\text{-}CH_3\text{-}H_4PteGlu$ arises as an intermediate in formation of the methyl group of methionine from $5,10\text{-}CH_2{=}H_4PteGlu$. The equilibrium constant is 2000-4000 and thus strongly favors the formation of $5\text{-}CH_3\text{-}H_4PteGlu$ (Katzen and Buchanan, 1965). The reverse reaction can be greatly accelerated by electron accepting compounds such as menadione (Donaldson and Keresztesy, 1962). These compounds presumably reoxidize $FADH_2$ produced in the reaction,

while under physiological conditions the reaction is practically irreversible and recycling of $H_4$PteGlu can only take place as a result of a homocysteine-dependent transmethylation reaction (Herbert and Zalvsky, 1962). This reductase is present in the livers of various vertebrates (Katzen and Buchanan, 1965; Donaldson and Keresztesy, 1962; Kisliuk, 1963; Kutzbach and Stokstad, 1967a), and in certain bacteria (Hatch *et al.*, 1961; Cathou and Buchanan, 1963; Kisliuk, 1963). The highly purified enzyme has a specific requirement for $FADH_2$ as reductant (Guest *et al.*, 1964; Foster *et al.*, 1964; Katzen, 1964). NADH can serve as reductant only when present with FAD and lipoamide dehydrogenase (Katzen and Buchanan, 1965). However, it is generally agreed that FAD is bound to the enzyme *in vivo*.

The 5,10-methylenetetrahydrofolate reductase reaction leads, as mentioned earlier, to the synthesis of methyl groups which can subsequently be transferred to homocysteine to form methionine (see below). $H_4$PteGlu is then regenerated. It is not surprising that these two reactions are strictly regulated in several species. For example, regulation of 5,10-methylenetetrahydrofolate reductase by L-methionine in *E. coli* has been observed (Rowberry and Woods, 1961). Detailed enzymatic studies by Katzen and Buchanan (1965) showed that synthesis of this reductase in *E. coli* mutants auxotrophic for methionine and cobalamin is controlled by an end-product repression mechanism. When no cobalamin was supplied in the growth medium, synthesis of the enzyme, as

reflected by the activity of crude extracts, was decreased by raising the methionine concentration in the medium. Similarly in methionine-free media, enzyme synthesis was repressed when the concentration of cobalamin was elevated, presumably because under these conditions the intracellular concentration of methionine is augmented as a result of increased synthesis of methionine.

Dickerman and Weissbach (1964) have shown that in the methionine-cobalamin auxotroph, *E coli* $K_{12}$ 2276, although synthesis of 5,10-methylenetetrahydrofolate was repressed by high levels of exogenous methionine, synthesis of the enzyme was greatly stimulated by low levels of methionine. Under the latter conditions, growth of the organism was significantly inhibited, presumably because the excessive amount of 5,10-methylenetetrahydrofolate reductase resulted in depletion of $H_4$PteGlu and 5,10-$CH_2$=$H_4$PteGlu by conversion to 5-$CH_3$-$H_4$PteGlu, with consequent depletion of HCO-$H_4$PteGlu to a degree that limited purine formation. This interpretation receives support from the fact that guanosine relieves the growth inhibition occurring under these conditions. In agreement with this, the addition of glycine, thymidine, inosine, guanosine or histidine did not cause repression of the enzyme in the absence of methionine (Taylor *et al.*, 1966).

Kutzback and Stokstad (1967b) have recently suggested that S-adenosylmethionine might act as a feedback inhibitor of 5,10-methylenetetrahydrofolate reductase *in vivo* from

their studies of the rat liver reductase. It is interesting to note that they were not able to detect repression of 5,10-methylenetetrahydrofolate reductase in rat tissues. The enzyme readily lost its susceptibility to S-adenosylmethionine and in other studies it was clear that S-adenosylhomocysteine could reverse the inhibition caused by S-adenosylmethionine. They, therefore, proposed the hypothesis that inhibition is produced by binding of S-adenosylmethionine at an allosteric site, for which S-adenosylhomocysteine competes. Repression of 5,10-methylenetetrahydrofolate reductase by high concentrations of PteGlu has been observed in *L. casei* (Ohara and Silber, 1969). To the author's knowledge the regulation of this enzyme in *Saccharomyces* has not been examined.

*5-Methyltetrahydrofolate-homocysteine transmethylase*

Two distinct reactions, one involving vitamin $B_{12}$ and the other proceeding without this vitamin, have been described. In the vitamin $B_{12}$ system, 5-$CH_3$-$H_4$PteGlu tri- or monoglutamate are effective methyl donors to homocysteine and vitamin $B_{12}$, in addition, catalytic amounts of SAM are required:

$$\text{5-CH}_3\text{-H}_4\text{PteGlu-tri-(mono-)glutamate + homocysteine} \xrightarrow[\text{SAM}]{\text{B}_{12}\text{ coenzyme}} \text{H}_4\text{PteGlu + methionine}$$

In the non-vitamin $B_{12}$ system, only the di- or triglutamate of 5-$CH_3$-$H_4$PteGlu are effective substrates. Vitamin $B_{12}$ and SAM are not required:

$5\text{-}CH_3\text{-}H_4PteGlu\text{-}tri\text{-}(di\text{-})glutamate + homocysteine \longrightarrow H_4PteGlu + methionine$

The vitamin $B_{12}$ system has been observed in mammalian liver extracts (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962) and both systems have been observed in *E. coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* (Woods *et al.*, 1965; Morningstar and Kisliuk, 1965). When cobalamin was added to the growth media of bacteria having both systems, the vitamin $B_{12}$ independent pathway was repressed (Morningstar and Kisliuk, 1965). $H_4PteGlu$ also inhibited the cobalamin independent system by competition with $H_4PteGlu_3$ for 5,10-methylenetetrahydrofolate reductase (Guest and Wood, 1965). This inhibition may clearly play a role in regulating methionine biosynthesis. Yeast has no vitamin $B_{12}$ (Jukes and Williams, 1954) and methylation of homocysteine is achieved by the non-$B_{12}$ pathway in which both the di- and triglutamate forms of $5\text{-}CH_3\text{-}H_4PteGlu$ are transmethylated (Burton *et al.*, 1969). Regulation of this enzyme in yeast has not yet been reported. However, considerable product inhibition of this enzyme activity has been observed in plants (Dodd and Cossins, 1970).

Another transmethylase leading to synthesis of methionine, but not deriving methyl groups directly from the pteroylglutamate pool, is shown in the following reaction:

Homocysteine + adenosylmethionine (or methyl methionine) $\longrightarrow$ methionine + adenosylhomocysteine

This enzyme occurs in plants (Dodd and Cossins, 1970) in *E. coli* (Balish and Shapiro, 1967) and *Saccharomyces cerevisiae* (Shapiro *et al.*, 1965). The enzymes from plants and *E. coli* are stimulated by low concentrations of methionine but inhibited at high concentrations (Dodd and Cossins, 1970; Balish and Shapiro, 1967). In *Saccharomyces cerevisiae*, the properties of this enzyme have been described. The Michaelis constants for S-adenosylmethionine and homocysteine are $8.6 \times 10^{-4}$M and $3.2 \times 10^{-4}$M, respectively. Enzyme activity is inhibited by the products methionine and S-adenosylhomocysteine. Product inhibition of the reaction is highly specific with regard to the methionine molecule. The hypothesis of an ordered binding in the reaction with homocysteine, the first substrate bound, and methionine, the last product released, has been suggested (Shapiro *et al.*, 1965). Pigg *et al.* (1962) found that the transmethylase enzyme system was lacking in certain methionine-requiring strains of *Saccharomyces cerevisiae*. Methionine repressed its synthesis.

One of the ultimate fates of methionine arising from these reactions, is its utilization in S-adenosylmethionine biosynthesis. S-adenosylmethionine is an active methyl donor and functions as such in at least sixty different metabolic reactions (Meister, 1965; Lederer, 1965). These include the biosynthesis of methionine (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962; Guest *et al.*, 1962; Kisliuk, 1963), the methylation of t-RNA (Mandel and Borek,

1961), DNA (Gold and Hurwitz, 1961) and lipids (Bremer and Greenberg, 1961). The reaction mechanism for SAM synthesis, based on studies with partially purified enzymes, was found to be similar in rabbit liver (Cantoni and Durell, 1957), baker's yeast (Mudd and Cantoni, 1958), barley seedlings (Mudd, 1960) and *E. coli* (Tabor and Tabor, 1960). The reaction had a requirement for methionine, $Mg^{2+}$ ions and ATP:

$$\text{Methionine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{SAM} + \text{pyrophosphate} + P_i$$

The reaction strongly favors the formation of SAM. In this connection, yeast is known to have high capacity to synthesize SAM. For example, 20-50 µmoles of SAM can be isolated from one gram of yeast incubated with L-methionine (Schlenk, 1965). Much of this SAM is accumulated in the vacuole of the yeast cell (Schlenk, 1965) and is apparently metabolically inert. The question of whether SAM in yeast plays a role in regulation of methionine biosynthesis is still to be ascertained.

As homocysteine is a precursor of methionine, regulation of methionine synthesis may be achieved within the pathway leading to homocysteine formation. In higher plants, fungi and bacteria, homocysteine is formed as a result of a transsulphuration reaction (Thompson, 1967). In *Neurospora*, Kerr and Flavin (1970) have shown that formation of homocysteine involves acetylation of homoserine, catalyzed by homoserine transacetylase. Replacement of the acetyl group by cysteine, formed by the progressive reduction of inorganic sulphur,

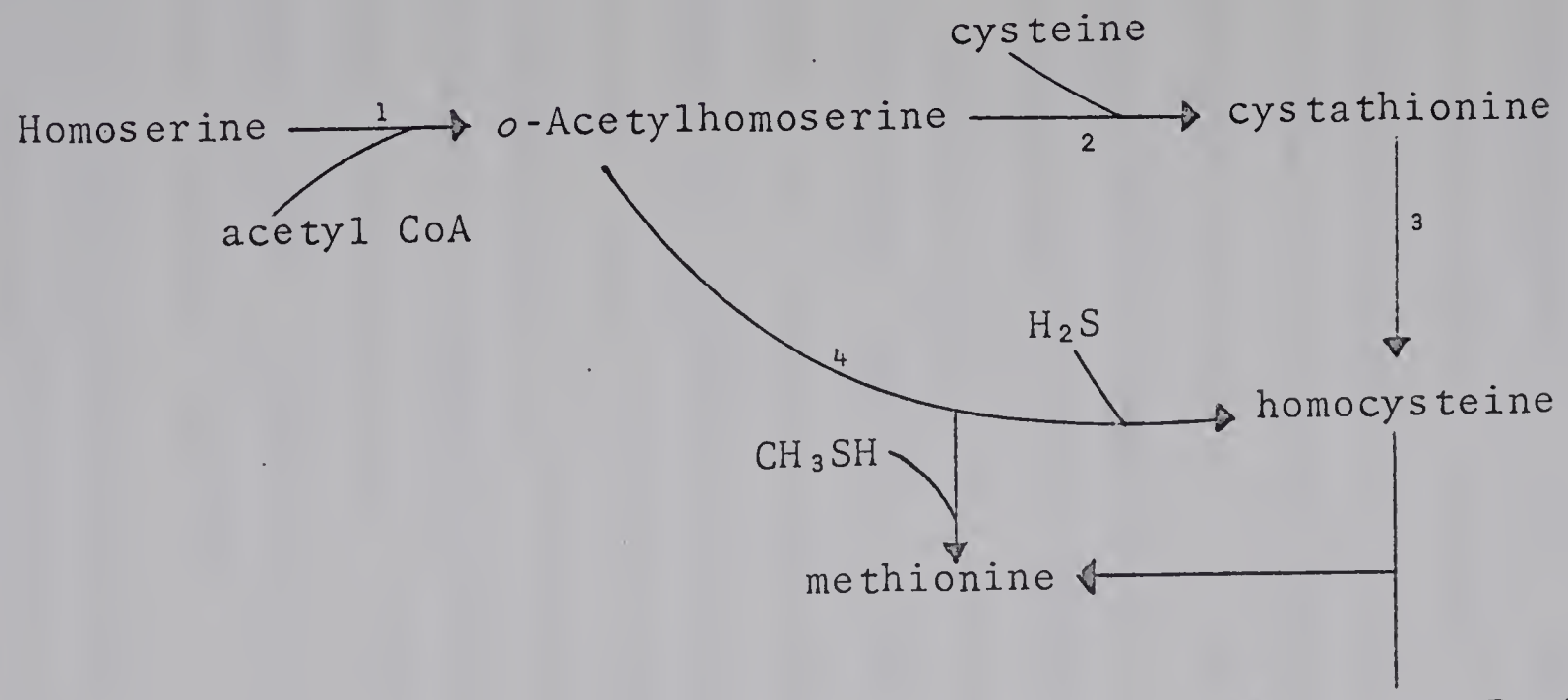
is catalyzed by cystathionine γ-synthase yielding cystathionine as an intermediate. By the action of β-cystathionase, homocysteine is formed from cystathionine (Scheme 2). Another enzyme (reaction 4, Scheme 2) catalyzes a reaction between acetylhomoserine and sulfide or methylmercaptan to yield homocysteine or methionine directly. However, this enzyme, acetylhomoserine sulfhydrylase, does not play a major role in methionine synthesis in this organism. Of possible significance in regulation of these reactions is the finding that the activity of cystathionine synthase is inhibited by S-adenosylmethionine (Kerr and Flavin, 1970). In yeast, only very low levels of cystathionine synthase have been detected (Kerr and Flavin, 1969). However, the formation of cystathionine from homocysteine and serine, catalyzed by cystathionine β-synthetase, has been reported in *Saccharomyces* (Delavier-Klutchko and Flavin, 1965). Cherest *et al.* (1969) have obtained results suggesting that in yeast the sulphydrylase reaction (reaction 4, Scheme 2) may be on the major route of methionine synthesis. The reaction was absent from a methionine auxotroph, and synthesis of the enzyme was repressed by methionine.

*10-Formyltetrahydrofolate synthetase* (Formate (ADP): tetrahydrofolate ligase E.C. 6.3.4.3)

This enzyme catalyzes the formation of 10-HCO-$H_4$PteGlu from $H_4$PteGlu and formate:

$$H_4PteGlu + ATP + HCOOH \rightleftharpoons 10\text{-}HCO\text{-}H_4PteGlu + ADP + P_i$$

SCHEME 2. GENERAL PATHWAY OF HOMOCYSTEINE FORMATION AND METHIONINE BIOSYNTHESIS




| Reaction No. | Enzyme |
|---|---|
| 1 | homoserine transacetylase |
| 2 | cystathionine γ-synthase (E.C. 4.2.1.13) |
| 3 | β-cystathionase (E.C. 4.2.1.15) |
| 4 | acetylhomoserine sulfhydrylase |

Several bacterial species utilizing purines for growth (Rabinowitz and Pricer, 1962; Whiteley *et al.*, 1959) have high levels of formyltetrahydrofolate synthetase. In other bacterial species, this enzyme activity is low or not detectable (Whiteley *et al.*, 1959; Albrecht and Hutchinson, 1964), but is present in almost all mammalian tissues which have been investigated (Whiteley, 1960). The importance of this enzyme in cell metabolism has not yet been clearly established, particularly as 10-HCO-$H_4$PteGlu can also be generated by the oxidation of 5,10-$CH_2$=$H_4$PteGlu by 5,10-methylenetetrahydrofolate dehydrogenase.

Several reports have been made regarding the regulation of this enzyme. Whiteley (1960) reported its induction by formate in *M. aerogenes* and Albrecht and Hutchison (1964) reported its repression by adenine, guanine and hypoxanthine in an anti-folate resistant mutant of *Streptococcus faecalis*. The *Saccharomyces cerevisiae* ad3 mutation which results in a requirement for adenine and histidine is associated with a decrease in the activity of this enzyme (Lazowska and Luzzati, 1970). Regulation of enzyme activity by methionine has not yet been reported.

*Serine hydroxymethyltransferase* (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase E.C. 2.1.2.1)

This enzyme catalyzes the following reaction:

$$\text{Serine} + H_4\text{PteGlu} \xrightleftharpoons{\text{pyridoxal phosphate}} \text{glycine} + 5,10\text{-}CH_2\text{=}H_4\text{PteGlu}$$

It has been detected in bacteria (Wright, 1955), plants (Cossins and Sinha, 1966) and in animal tissues (Blakley, 1954). The reaction is thought to be the first step in the biogenesis of the methyl group of methionine in mammalian liver (Elford *et al.*, 1965), in *E. coli* (Taylor and Weissbach, 1965) and in yeast (Pigg *et al.*, 1962; Botsford and Parks, 1969). It is also thought to be the first reaction in the synthesis of the majority of one-carbon fragments at the oxidation levels of formaldehyde and formate (Mudd and Cantoni, 1964).

Only preliminary information is available on the control of this enzyme. Nurmikko *et al.* (1964) have reported that the addition of glycine, DL-serine, DL-threonine, pteroyl-L-glutamate, sarcosine, adenine and xanthine to growing cultures of *E. coli* did not affect the activity of this enzyme. In *Saccharomyces*, Botsford and Parks (1969) have shown that methionine and S-adenosylmethionine inhibited the activity of serine hydroxymethyltransferase. The physiological significance of this inhibitory effect is not clear particularly if serine is an important precursor of thymidylate.

*The Present Investigation*

It is clear from the above review of literature pertaining to various aspects of one-carbon metabolism that information regarding control mechanisms is still incomplete.. As emphasized in this review it is clear that

pteroylglutamates are involved in the biogenesis of methionine, a compound of considerable physiological significance as it is a direct precursor of SAM. In *E. coli*, biosynthesis of methionine appears to be regulated through repression of methylenetetrahydrofolate reductase by methionine (Rowberry and Woods, 1961) while in mammals, this synthesis is regulated by end-production inhibition of enzyme activity. Thus, SAM inhibits the transfer of methyl groups from the pteroylglutamate pool by its effect on methylenetetrahydrofolate reductase (Kutzbach and Stokstad, 1967). Although the pathways for synthesis of methionine and SAM have recently been elucidated for *Saccharomyces*, it is still not clear what mechanisms regulate the flow of one-carbon units from the pteroylglutamate pool. An elucidation of these mechanisms would be of particular interest in the wild type which has no exogenous requirement for methionine but readily synthesizes SAM when this amino acid is supplied. The question, therefore, arises as to whether cells devoid of an exogenous supply of methylated compounds have an altered one-carbon metabolism. In a regulatory sense, it is conceivable that when methionine is available in the medium, the biosynthesis of this amino acid would not be required and the levels of related pteroylglutamates might be altered possibly as a result of repression or inhibition of key enzymes of one-carbon metabolism. Preliminary experiments in this area by the author prompted the more detailed investigations reported in this thesis.

The present work has examined the levels of pteroylglutamates during growth of yeast in the presence and absence of exogenous L-methionine. Considerable alterations in the pool size of pteroylglutamates were observed under these culture conditions. Studies of the key enzymes of one-carbon metabolism demonstrated that L-methionine affected, in certain cases, the levels and activities of the enzymes studied. Further experiments using formate-$^{14}C$ supported the hypothesis that culture of yeast in the presence of L-methionine regulates the synthesis and transfer of methylated pteroylglutamates.

# MATERIALS AND METHODS

*Chemicals*

Pteroylglutamic acid, tetrahydropteroylglutamic acid and D,L-homocysteine thiolactone were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Amino acids, nitrogenous bases and nucleosides, were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-*bis*-[2-(5-phenyloxazolyl)]benzene (POPOP) were purchased from Nuclear-Chicago, Des Plaines, Illinois. Special enzyme grade ammonium sulphate was obtained from Mann Research Laboratory, Inc., New York. All other chemicals were purchased from Fisher Scientific Company, Edmonton.

Pteroylglutamic acid-2-$^{14}$C, 5-methyl-$^{14}$C-tetrahydropteroylglutamic acid, S-adenosyl-L-methionine (methyl-$^{14}$C) and sodium formate-$^{14}$C were purchased from the Radiochemical Centre, Amersham, England. Pteroylglutamic acid-2-$^{14}$C was dissolved in sterile demineralized water to give a final specific radioactivity of 1 µc/0.02 µmole/1.7 ml of solution. 5-Methyl-$^{14}$C-tetrahydropteroylglutamic acid was dissolved in 0.5% potassium ascorbate solution (pH 6.0) to give a final specific radioactivity of 1 µc/0.016 µmole/0.8 ml of solution. Sodium formate-$^{14}$C was dissolved in sterile demineralized water to give a final specific radioactivity of 1 µc/0.023 µmole/0.2 ml of solution and S-adenosylmethionine (methyl-$^{14}$C) was dissolved in diluted $H_2SO_4$ (pH 3.0) to

give a final specific radioactivity of 1 µc/0.017 µmole/0.1 ml of solution.

*Preparation of stock solutions for yeast culture media*

The media for growth of yeast cultures were routinely prepared by combination of various stock solutions. These stock solutions were prepared essentially as described by Chan (1971) and contained the following components.

(a) Amino acid solution:

The composition of the amino acid solution is shown in Table 1. In this preparation, the amino acids were dissolved in approximately 40 ml of distilled water plus 10 mls of concentrated HCl. The solution was then diluted with water to 250 ml.

(b) Citrate buffer solution:

100 g of potassium citrate and 20 g of citric acid were dissolved in water and made up to 1 litre.

(c) Salt solution I:

17 g, KCl; 10.3 g, $MgSO_4 \cdot 7H_2O$; 100 mg, $FeCl_3 \cdot 6H_2O$ and 100 mg, $MnSO_4 \cdot H_2O$ were dissolved in about 800 mls of water to which 2 mls of concentrated HCl had been added. 100 mls of a solution containing 5 g, $CaCl_2 \cdot 2H_2O$ were then added followed by a final dilution with water to 1 litre.

(d) Salt Solution II:

22 g, $KH_2PO_4$ and 40 g, $(NH_4)_2HPO_4$ were dissolved in water and then made up to 1 litre.

(e) Vitamin solution I:

10 mg thiamine·HCl and 1 g inositol were dissolved in 200 mls of water, followed by dilution to 1 litre.

(f) Vitamin solution II:

200 mg calcium pantothenate and 200 mg nicotinic acid were dissolved in 200 mls of water. 8 mls of a biotin solution, prepared by dissolving 10 mg biotin in 100 mls 50% alcohol, were then added. The solution was then diluted with water to 1 litre.

*Preparation of yeast culture media*

The composition of the liquid medium is shown in Table 2. This basal media was adjusted to pH 4.5 with $(NH_4)_2HPO_4$ solution (50% w/v) as described earlier by Chan (1971). All sterilizations were performed in an autoclave at 15 lbs. psi. The culture media were maintained at this pressure for 15 minutes. The media were prepared not longer than 24 hours before use.

*Preparation of solid media for maintaining active yeast*

25 g of Bacto-wort agar (Difco Laboratories, Detroit, Michigan, U.S.A.) were suspended in 400 mls of water in a 500 ml Erlenmeyer flask. The flask was plugged with cotton and heated on a boiling water bath until the agar was completely dissolved. The volume was then adjusted to 500 ml with distilled water. A pipetting syringe was used to dispense the agar solution (10 mls) into fifty 20x150 mm test tubes. The tubes were subsequently plugged with

TABLE 1

COMPOSITION OF AMINO ACID SOLUTION

| Amino acids | gm/250ml |
|---|---|
| DL-leucine | 1.0 |
| L-isoleucine | 0.5 |
| DL-valine | 1.0 |
| L-cysteine | 1.0 |
| L-tryptophan | 0.5 |
| L-tyrosine | 0.5 |
| DL-phenylalanine | 1.0 |
| L-glutamic acid | 2.0 |
| DL-threonine | 1.0 |
| DL-alanine | 1.0 |
| L-aspartic acid | 1.0 |
| L-lysine HCl | 1.0 |
| L-arginine HCl | 0.5 |
| L-histidine $HCl \cdot H_2O$ | 0.5 |
| DL-serine | 1.0 |
| L-proline | 0.25 |
| glycine | 0.5 |

cotton. Following autoclaving for 15 minutes at 15 lbs. psi, they were cooled in a slanted position and stored at 2°C.

*Preparation of DEAE-cellulose columns*

Columns of DEAE-cellulose were prepared by the method of Sotobayashi *et al*. (1966). 18 g of N,N-DEAE-cellulose and 22.5 g Hyflo Supercel were suspended in 1.5 litres of distilled water. The slurry was packed into glass columns (2.0 cm) by gravity flow to a depth of 28 cm. The columns were washed with 50 mls of 0.5 N KOH followed by distilled water until the rinse had a pH value of 7.0-7.5. Fifty mls of 0.5 M potassium phosphate buffer (pH 6.0) were then added, followed by distilled water until the rinse was free of phosphate. The columns were kept at room temperature until required.

*Preparation of ion exchange resins*

Analytical grade cation exchange resin, Dowex 50Wx8 ($H^+$ form) 100-200 mesh and anion exchange resin, AG1-X10 ($Cl^-$ form) 200-400 mesh were obtained from Bio Rad Laboratories, Richmond, California. The cation exchange resin was first made into a thin slurry using distilled water and the fines removed by suction. This procedure was repeated several times. The wet resin, 100 ml bed volume, was then washed (4 washes of 100 mls) in an Erlenmeyer flask with 6N $H_2SO_4$ to ensure that it was in the $H^+$ form. After acid washing, the resin was rinsed several times in the flasks with distilled water until the pH of washing was at least

TABLE 2

COMPOSITION OF THE BASAL MEDIUM OF YEAST CULTURE

| Compounds | Quantity/litre |
|---|---|
| Citrate buffer | 100 ml |
| Amino acid solution | 100 ml |
| Vitamin solution I | 50 ml |
| Vitamin solution II | 25 ml |
| Salt solution I | 50 ml |
| Salt solution II | 50 ml |
| Glucose | 100 gm |

5.0. Resin required in the sodium form received an additional washing with 800 ml of 4N NaCl followed by 200 ml of 0.1 N NaCl, these volumes being necessary to convert 100 ml of wet resin from the $H^+$ to $Na^+$ form. The resins were then stored at 4°C until required.

*Culture of Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* (ATCC 9763) was purchased from American Type Culture Collection, Rockville, Maryland, U.S.A., and suspended in physiological sterilized saline (0.9% w/v NaCl). A small quantity of the suspended yeast was then transferred with the aid of a sterilized transfer loop to wort agar slants. The slants were incubated at 30°C until a visible line of growth appeared. Transfer was then made onto new slants and the incubation repeated. Subsequent transfers were made once a week to maintain active yeast cells.

Inocula of actively growing cells were transferred from the slants into 100 mls of the basal medium (Table 1) containing a supplement of L-methionine (2.5 µmole/ml) contained in a 250 ml Buchner flask. The side arm of the flask was connected to the vacuum line and sterilized air was drawn into the culture through a fine sintered glass aerator. After a growth period of 24 hours at 30°C, the cells were harvested by centrifugation at 4000 x g for 10 minutes in a Servall Refrigerated Automatic Centrifuge operated at 2°C. The cells were washed twice with cold sterile demineralized water. The washed yeast was then

suspended in 10 mls of cold sterile demineralized water and this served as the inoculum for experimental cultures. Five percent of this inoculum was added to 200 ml of the basal medium without addition of L-methionine, or with various concentrations of this amino acid. The cultures were then incubated at 30°C with aeration as described above.

*Measurement of cell growth*

The growth of yeast cells in different culture media was followed spectrophotometrically using a Beckman DB-G spectrophotometer. Optical density at 550 nm was measured throughout the growth period.

*Extraction of pteroylglutamate derivatives*

Pteroylglutamate derivatives were extracted from yeast cells by the method of Bird *et al.* (1965), with slight modifications. At the end of the culture period, the cells were harvested as described earlier and washed three times with cold sterile demineralized water. The washed cells were then suspended in 2 mls of 1% (w/v) potassium ascorbate (pH 6.0) and lyophilized (Virtis Automatic Freeze-Dryer, Model 10-010). The lyophilized cells were weighed, suspended in 2 mls of 1% (w/v) potassium ascorbate (pH 6.0) and sonicated for 5 minutes in a Fisher Ultrasonic Generator (manufactured by Blackstone Ultrasonic Inc., Model SSO) at 2°C.

After sonication, 3 mls of 1% potassium ascorbate (w/v), pH 6.0, were added to the homogenates. The homogenates

were heated for 10 minutes by immersion in a water bath maintained at 95°C and cooled rapidly in an ice bath. Cell debris and denatured protein were removed by centrifugation at 18,000 x g for 20 minutes. The supernatant was diluted to 10 mls with 1% (w/v) potassium ascorbate (pH 6.0).

*Chromatography of pteroylglutamate derivatives*

Pteroylglutamate derivatives present in the yeast extracts were separated by DEAE-cellulose column chromatography (Sotobayashi *et al.*, 1966). Aliquots (1-5 mls) of the extracts, containing 0.4-1.0 µg of pteroylglutamates, were applied to the columns and washed into the cellulose bed with two 2 ml portions of 0.6% (w/v) potassium ascorbate (pH 6.0). Five mls of this ascorbate solution were then placed on the column. 0.5 M potassium phosphate buffer (pH 6.0), containing 0.6% (w/v) potassium ascorbate, was then passed dropwise through a mixing chamber which contained 200 mls of 0.6% (w/v) potassium ascorbate (pH 6.0). Fractions of 3 mls were collected from the column at room temperature in tubes containing an additional 0.1 ml of 10% (w/v) potassium ascorbate (pH 6.0) using an LKB Ultra Rac Fraction Collector, Type 700 (LKB-Produkter AB, Stockholm, Sweden). Following collection of fraction 130, the gradient elution was stopped and the column further eluted with 0.5 M potassium phosphate (pH 6.0) containing 0.6% (w/v) ascorbate. The fractions were either assayed immediately with *L. casei* and *P. cerevisiae* or were stored in a frozen state until required. The elution sequence of various common folate derivatives

was determined by chromatography of 0.05-0.10 µg samples of the authentic derivatives under these conditions.

*Microbiological assay*

The levels of pteroylglutamates in yeast extracts or in fractions collected during DEAE-cellulose column chromatography were measured by the 'aseptic plus ascorbate' method of Bakerman (1961). *Lactobacillus casei* (ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081), purchased from the American Type Culture Collection, Rockville, Maryland, U.S.A., were used as the assay bacteria. As these bacteria require pteroylglutamates for growth in defined media, the level of pteroylglutamates could be determined by measurement of growth (Jukes and Stokstad, 1948; Hansen, 1964; Freed, 1966). Bacterial growth was measured by titration of the lactic acid produced after 72 hours incubation at 37°C (Roos, 1971). Reference curves were constructed using authentic PteGlu for *L. casei* and 5-HCO-$H_4$PteGlu for *P. cerevisiae*.

*Assay of polyglutamyl derivatives*

Because pteroylglutamyl derivatives with more than three glutamyl residues do not support the growth of either *L. casei* or *P. cerevisiae*, assay of such derivatives involved hydrolysis using a γ-glutamyl carboxypeptidase (Blakley, 1969). γ-Glutamyl carboxypeptidases from chicken pancreas (Mims and Laskowski, 1945), from hog kidney (Doctor and Couch, 1953) and from 3-day-old pea cotyledons (Roos and Cossins, 1971), were routinely used in these studies. The chicken pancreas

enzyme was isolated from acetone powders (Difco Laboratories) as described by Mims and Laskowski (1945), and that from hog kidney was prepared from a commercial extract purchased from Difco Laboratories following the method of Doctor and Couch (1953). The pea cotyledon hydrolase was prepared as described by Roos and Cossins (1971). The reaction mixture for the avian enzyme (see Results Section) contained 0.2 M sodium borate buffer (pH 7.8) and 0.1 M calcium chloride. The reaction mixtures for the other two enzymes included 0.1 M sodium acetate buffer (pH 4.5) containing 10% (w/v) ascorbate. Enzyme activities were routinely checked by using Difco-Bacto yeast extract as substrate (Roos and Cossins, 1971). Enzyme activities were confirmed in all cases by including reaction systems containing boiled enzyme.

*Sodium formate-$^{14}C$ feeding experiments*

In all feeding experiments, cells were cultured for 24 hours in the basal culture medium which in some cases was supplemented with L-methionine. The cells were then harvested and transferred to 50 mls of new basal culture media, containing 2.5 µmoles L-methionine/ml or without methionine respectively. These culture flasks were plugged with cotton and aeration was supplied by a rotary shaker at 30°C. 2.5 µCi of sodium formate-$^{14}C$ (1 µCi/0.023 µmole) were added after 3 hours of growth at which time the cells were in the logarithmic phase. Growth was continued for various periods (see Results Section), the cells were

harvested as before and washed three times with cold sterile demineralized water to remove exogenous radioisotope. Uptake of the radioactive compounds was calculated from the amounts of radioactivity left in the media. The cells were then lyophilized in the presence of 1% potassium ascorbate (pH 6.0) and weighed. Extraction of pteroylglutamates from the dried cells was the same as described earlier in this thesis. When cells were required for the assay of S-adenosylmethionine, the washed cells were subjected to perchloric acid extraction (see below) without lyophilization.

*Assay of S-adenosylmethionine*

At the end of the culture period, the yeast was harvested and washed with cold sterile demineralized water, and S-adenosylmethionine was isolated by the method described earlier (Shapiro and Ehninger, 1966). The cells were suspended in 2 mls of 1.5 N perchloric acid. The acid suspension was then sonicated for 5 minutes at 4°C. After centrifugation at 10,000 x g for 10 minutes the residue was washed with about 3 mls of ice cold demineralized water. The pH of the combined supernatants was then adjusted to 6.3 by addition of solid $KHCO_3$. After a further centrifugation at 10,000 x g for 10 minutes, the supernatant was divided into two equal parts, each of which was added to columns of $Na^+$ and $H^+$ resin, respectively.

When $H^+$ resin was used, the extracts were mixed with 6 mls of wet resin in a 125 ml Erlenmeyer flask and agitated at intervals for 20 minutes at 4°C. The extract and resin

were then poured into columns (1.2 cm diameter) to a depth of 6 cms.

Isolation of SAM by column chromatography was a slight modification of the method described by Shapiro and Ehninger (1966). The columns containing the extracts were routinely eluted at room temperature with 100 mls of demineralized water, followed by 100 mls of 2N HCl and finally 100 mls of 6N HCl. The 6N fractions from the $Na^+$ columns contained only SAM. S-adenosylhomocysteine (SAHC) was collected in the 2N HCl fractions. The 6N fractions from the $H^+$ columns contained both SAM and SAHC. For quantitative analysis of SAM, 6N fractions from the $Na^+$ column were assayed spectrophotometrically using a Beckman DB-G spectrophotometer. The molar extinction coefficients for SAM in 6N acid eluates were taken as $E_m$ = 14,700 at 260 nm (Shapiro and Ehninger, 1966). For assays of radioactivity in 2N and 6N eluates, the eluating acid was completely removed *in vacuo* at 40°C. The residue was then redissolved in distilled water, and the aqueous aliquots were counted by liquid scintillation without the high degree of quenching normally encountered with strongly acidic solutions.

*Degradation of S-adenosylmethionine*

The SAM resulting from formate-$^{14}C$ feeding experiments was purified by phosphotungstic acid precipitation (Schlenk and Depalma, 1957) and the SAM was reisolated by chromatography on Dowex 50W-X8 ($Na^+$ form) resin. The SAM was hydrolyzed with 0.1 N NaOH at the temperature of a boiling

water bath for 5 minutes to give adenine and methionine as the major products (Parks and Schlenk, 1958). The hydrolysates were identified by thin layer chromatography.

*Thin layer chromatography and autoradiography*

Thin layer chromatography, using mixed layers of silica gel GF254 and cellulose powder MN300, was employed mainly for the identification of S-adenosylmethionine and its degradation products. Silica gel GF254 and cellulose powder were obtained from Canadian Laboratory Supplies, Edmonton. The mixed layers were prepared by homogenizing 12 gm of cellulose powder with 6 gm of silica gel GF254 in 100 mls of demineralized water using a Virtis homogenizer operated at 10,000 rpm for 60 seconds. Layers (300 μ thick) were then spread onto 20 cm x 20 cm glass plates using a Desaga spreader (Desaga, Heidelberg, West Germany). The layers were allowed to stand for 20 minutes to allow evaporation of surface moisture. The plates were then dried horizontally in an oven at 90°C-100°C for one hour and stored in a cabinet containing a silica gel desiccant.

Thin layer chromatography was routinely carried out using *n*-butanol:glacial acetic acid (99.7%):water (20:15:25 v/v/v), *n*-propanol:water (70:30 v/v) or *n*-propanol:ammonium hydroxide (0.90 sp. gravity):water (70:9:21 v/v/v) as solvent systems. The $R_f$ values of the compounds separated are given in the appropriate parts of the Results section.

Labelled compounds were detected on thin layer plates by autoradiography using Kodak "No Screen" X-ray film. Areas

on the plates containing 600 dpm of $^{14}C$ could be readily detected after 5 days exposure. The exposed films were developed using G.E. Supermix X-ray developers and fixer (General Electric X-ray Company, Milwaukee, Wisconsin, U.S.A.).

*Counting of radioactive samples.*

Radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Corp., Unilux II model). For aqueous samples, a dioxane-based fluor system was used. This fluor contained 12.0 gm of PPO and 0.5 gm of POPOP to each litre of a mixture of dioxane:anisole:dimethoxyethane (6:1:1 by volume). Usually 0.1 - 0.5 ml of the labelled sample were added to 15 mls of fluor. A counting efficiency of approximately 65% was obtained as determined by the channels ratio method.

Radioactive areas on thin layer chromatograms detected by autoradiography were carefully removed with a scalpel blade and placed in scintillation vials containing 15 mls of a toluene counting solution and filled to approximately one-third with dry Cabosil. The toluene counting solution contained 12.0 gm of PPO and 0.5 gm POPOP to each litre of toluene. Counting efficiency by the channels ratio method was 72%.

*Analysis of yeast amino acids*

Levels of free amino acids and ammonia in yeast extracts were determined using a Beckman Automatic Amino Acid Analyzer, Model 121. For such analyses, cells grown in

the basal medium for 24 hours, as described earlier, were harvested and transferred to 500 mls of new culture media containing 2.5 µmole L-methionine/ml or without methionine respectively. The cultures were incubated at 30°C under aerobic conditions. Cells present in 100 ml aliquots of the culture medium were harvested at various periods by centrifugation, washed and resuspended in 2 mls of cold demineralized water. The suspensions were then sonicated and heated at 95°C in a boiling water bath for 10 minutes. Denatured protein and cell debris were removed by centrifugation at 18,000 x g for 10 minutes. After centrifugation, the supernatants were passed through columns (1x6 cm) of Dowex 50W-X8 ($H^+$ form) which were then washed by 50 mls of demineralized water and 50 mls of 2N HCl. The 2N HCl effluent containing the amino acids were collected and dried *in vacuo* at 40°C. The dried residue was finally dissolved in 2 mls of 0.2 M citrate buffer (pH 2.2). Aliquots of this amino acid extract were then subjected to analysis in the Amino Acid Analyzer using UR-30 and PA-35 spherical resins (Beckman Instruments Inc., California, U.S.A.). The eluting buffer for separating the neutral and acidic amino acids was 0.20 M citrate at pH 3.22 and 4.25. The basic amino acids were eluted from the PA-35 resin using 0.35 M citrate (pH 5.25). The concentration of each amino acid present was determined by the HXW method. The pH values of all buffers were measured at 22°C.

For determination of radioactive amino acids, the 2N HCl

effluent from the Dowex 50W-X8 $H^+$ column was dried *in vacuo* at 40°C and redissolved in 1 to 2 mls of 0.2 M citrate buffer (pH 2.2). Aliquots of this was subjected to amino acid analysis. The amino acids were eluted by the buffers mentioned above but in this case the effluent was collected in fractions of 2.8 mls using a fraction collector. The elution pattern of the amino acids was determined by using an authentic amino acid mixture as reference. The collected fractions were reacted with ninhydrin in the fractions collected to confirm the elution sequence of different amino acids. For further confirmation, the amino acid extracts were co-chromatographed with authentic amino acids and analyzed by the same method.

*Experiments involving cell-free extracts*

(a) Preparation of cell-free extracts.

Cells, after 6 hour's growth, were harvested and washed as described earlier in this thesis. The washed cells were suspended in 5 mM potassium phosphate buffer (pH 6.9) containing 5 mM 2-mercaptoethanol and sonicated for 5 minutes at 4°C. After centrifugation at 18,000 x g for 20 minutes, the supernatant was assayed for the following enzyme activities.

(b) 10-HCO-$H_4$PteGlu synthetase

The reaction system for the assay of formyltetrahydropteroylglutamate synthetase activity (Hiatt, 1965) contained 100 µmoles triethanolamine buffer (pH 8.0), 150 µmoles Tris formate (pH 8.0), 2.5 µmoles $MgCl_2$, 200 µmoles KCl, 4 µmoles

DL-$H_4$PteGlu (Sigma Chemical Company, U.S.A.), 2 µmoles of ATP and 0.1 ml of cell-free extract in a total volume of 1 ml. The control systems contained all of these components with the exception of the ATP. The reaction mixture was incubated at 30°C for 10 minutes. The reaction was stopped by adding 1 ml of 1N HCl and allowed to stand for 10 minutes. The denatured protein was removed by centrifugation. Under these conditions, the 10-formyltetrahydropteroylglutamate formed in the reaction was converted to 5,10-methenyltetrahydropteroyl-glutamate. The latter compound was estimated spectrophotometrically at 355 mµ ($E_m$ = 22,000).

(c) Serine hydroxymethyltransferase

Activity of serine hydroxymethyltransferase was assayed by the isotopic method of Taylor and Weissbach (1965). Radioactivity in the C-1 unit of 5,10-$CH_2$=$H_4$PteGlu produced in the reaction, was trapped with carrier formaldehyde and converted to a dimedon addition product. The reaction system contained 30 µmoles phosphate buffer (pH 8.5), 1.0 µmole $H_4$PteGlu in 1.0 M 2-mercaptoethanol, 0.1 µmole pyridoxal-5'-phosphate, 0.1 µCi of DL-serine-3-$^{14}$C (1 µCi/0.16 µmole) and 0.1 ml cell-free extract in a total volume of 0.4 ml. All components except serine were first incubated for 5 minutes at 30°C. Reactions were then initiated by addition of the substrate and were terminated 15 minutes later by 0.3 ml of 1.0 M sodium acetate (pH 4.5), 20 µl of 1.0 M formaldehyde and 0.3 ml of 0.4 M dimedon (in 50% ethanol) added in succession. The reaction vessels were then

heated for 5 minutes in a boiling water bath to accelerate formation of the HCHO-dimedon derivative. The tubes were then cooled for 5 minutes in an ice bath. The dimedon compound was extracted by vigorous shaking with three 1 ml aliquots of toluene at room temperature. The aqueous toluene phases were separated by centrifugation, the upper phase being removed for measurement of $^{14}C$.

(d) 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase.

An isotopic assay (Dodd and Cossins, 1970) was used to measure the activity of this enzyme. The standard 0.5 ml assay mixture consisted of 0.1 ml of cell-free extract, 1 µmole of L-homocysteine, freshly prepared from the thiol-actone (Sigma Chemical Company, Missouri, U.S.A.), 0.1 µCi of 5-$^{14}CH_3$-$H_4$PteGlu (1 µCi/0.016 µmole) and 50 µmoles of potassium phosphate buffer (pH 6.9). Control systems contained all of these components with the exception of the homocysteine. The mixture was incubated at 30°C for 30 minutes and the reaction terminated by rapid cooling in an ice bath. The cooled reaction mixture was placed on a column (0.5 x 2.5 cm) of Dowex 1-X10 resin in the $Cl^-$ form. The column was eluted with six washings each of 0.2 ml distilled water. Under these conditions the labelled substrate was retained by the $Cl^-$ column while labelled methionine was quantitatively eluted and collected in a scintillation vial. The radioactivity in the reaction product was measured by liquid scintillation counting using 15 mls of the dioxane scintillation fluid. The amount of radioactivity in the

eluates was taken as a direct measure of transmethylase activity. The amount of methionine produced was calculated from the specific radioactivity of the 5-$CH_3$-$H_4$PteGlu.

(e) 5,10-$CH_2$=$H_4$PteGlu reductase.

The enzyme was assayed by the menadione-dependent oxidation of 5-$^{14}CH_3$-$H_4$PteGlu to $H_4$PteGlu and $^{14}$C-formaldehyde (Donaldson and Keresztesy, 1962). The latter compound being in equilibrium with the immediate product, 5,10-$CH_2$=$H_4$PteGlu. The reaction system for this assay contained 10 µmoles potassium phosphate buffer (pH 7.4), 5 mµmoles FAD, 5 mµmoles menadione, 5 µmoles formaldehyde, 0.1 µCi of 5-$^{14}CH_3$-$H_4$PteGlu (1 µCi/.016 µmole) and 50 µl of cell-free extract in the total volume of 0.32 ml. Control systems contained all of these components with the exception of the cell-free extract. The reaction systems were incubated at 30°C for 30 minutes and the reactions were stopped by rapid cooling in an ice bath. The cooled reaction mixture was immediately placed on a column (0.5 x 2.5 cm) of Dowex AG1-X10 resin ($Cl^-$ form). The column was eluted with three washings each of 0.5 ml distilled water. Under these conditions, 5-$^{14}CH_3$-$H_4$PteGlu was retained by the column and the product, $^{14}$C-formaldehyde, was eluted and collected in a scintillation vial. The radioactivity in this product was measured by liquid scintillation counting using 15 mls of the dioxane scintillation fluid. The amount of $^{14}$C-formaldehyde formed was calculated from the specific radioactivity of the 5-$CH_3$-$H_4$PteGlu.

*Estimation of protein*

The protein content of cell-free extracts was estimated colorimetrically using the method of Lowry *et al.* (1951). Crystalline egg albumin was used as a reference standard. All determinations were made at least in duplicate.

# RESULTS

## *Growth of Yeast Cells in Different Culture Media*

Before any attempts were made to study the effect of methionine on one-carbon metabolism in yeast, the effect of this amino acid on growth was studied. Growth was measured by measuring the optical densities of the cultures at 550 mμ. During a 24 hour growth period, aliquots were removed from the cultures at intervals and appropriate dilutions with water were made for optical density measurement.

Growth curves of yeast grown in the basal medium with and without methionine supplement (2.5 μmole/ml) are shown in Figure 1. An S shaped growth was observed. The logarithmic phase of growth started after a lag phase of 2-3 hours and proceeded until approximately the 17th hour in case of the methionine supplemented culture, and until approximately the 19th hour in case of the culture without methionine supplement. A post-exponential phase was obvious after approximately 20 hours in both cultures. It is clear that the cells cultured in the presence of methionine grew slightly more rapidly than those in the absence of this amino acid. However, the substantial growth observed in the absence of this amino acid indicates that the cells were capable of satisfying any requirement for this compound.

## *Effect of L-methionine on the Levels of Pteroylglutamate Derivatives*

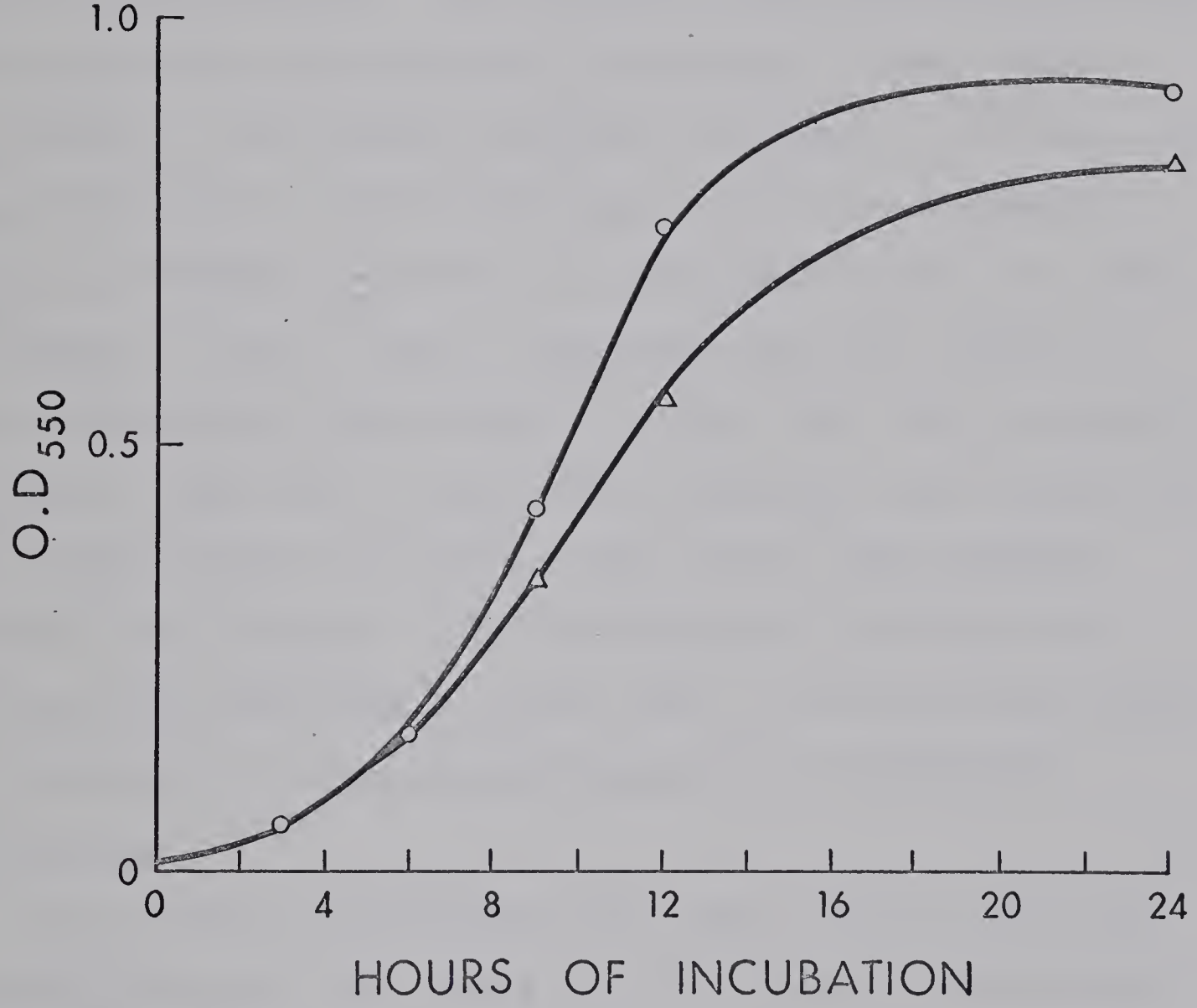
In these experiments the cells were grown in different

FIGURE 1

*GROWTH CURVES OF YEAST CELLS IN BASAL MEDIUM WITH AND WITHOUT L-METHIONINE SUPPLEMENT*

Cells were initially grown with aeration in the basal medium with L-methionine (2.5 mM). After 24 hours at 30°C, samples of the cells were transferred immediately to the new culture media with methionine supplement (o) or without methionine supplement (Δ). Aliquots were removed at intervals as indicated. All readings were made after a 10-fold dilution.

The data represent the mean of four separate experiments.


1.0
0.5
0
O.D 550
0
4
8
12
16
20
24
HOURS OF INCUBATION

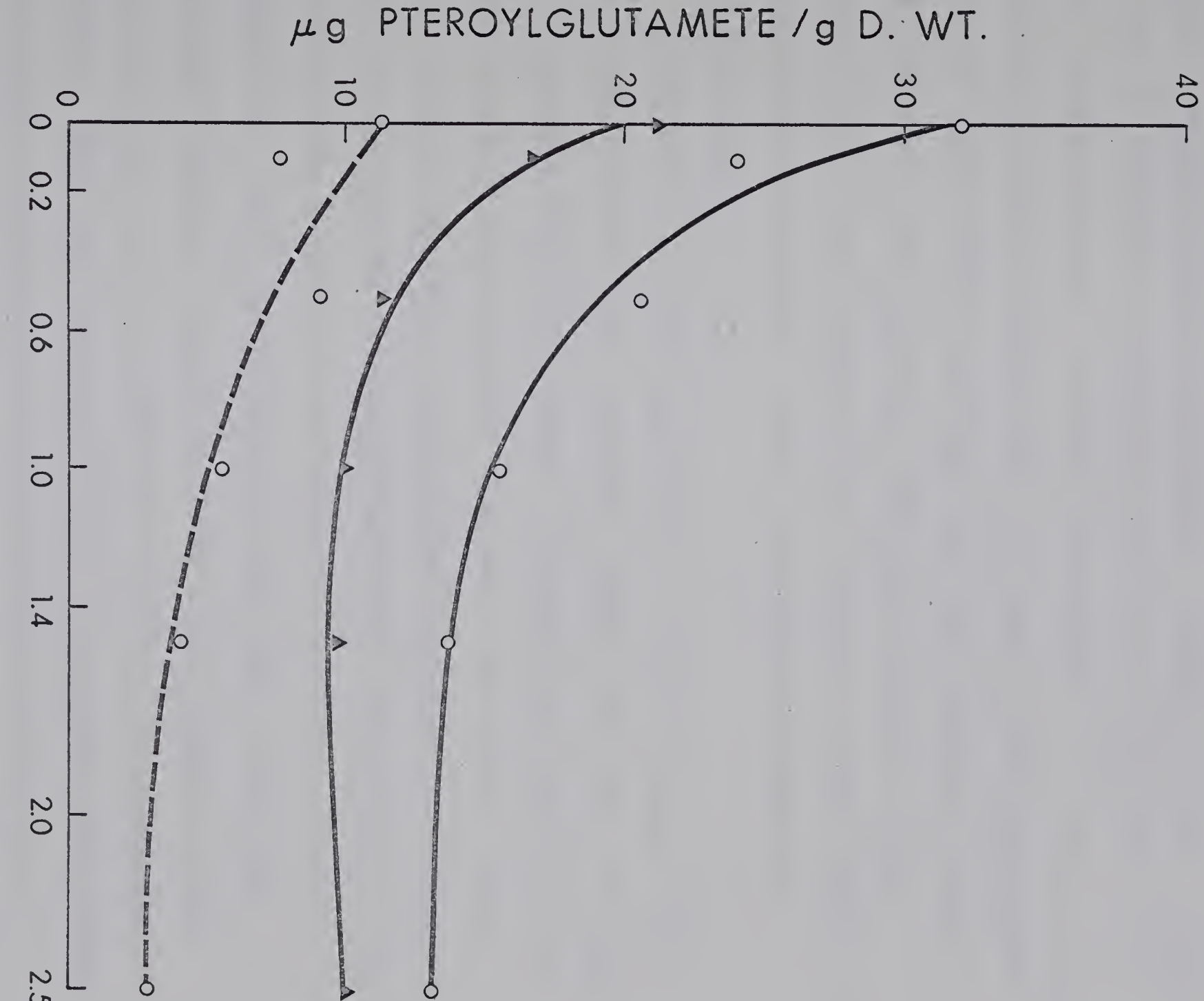
culture media supplemented with various concentrations of L-methionine (0-2.5 µmole/ml) at 30°C with aeration. After 6 hours of growth, optical densities of each culture were taken and pteroylglutamate derivatives were extracted in the presence of potassium ascorbate (pH 6.0) as described in the Materials and Methods. The levels of pteroylglutamates were then assayed microbiologically employing *L. casei* and *P. cerevisiae*. The results are given in Figure 2. As the test organisms used in the microbiological assay were unable to utilize naturally occurring pteroylpolyglutamates, the data in Figure 2, for *L. casei*, represents only the levels of pteroylglutamates which contain no more than three glutamyl residues. Data for *P. cerevisiae* represents only formylated and unsubstituted derivatives with two or less glutamyl moieties per molecule. The difference in the levels of derivatives found when *L. casei* and *P. cerevisiae* were used may be taken as an approximate measure of methylated derivatives.

It is clear from Figure 2 that the highest levels of pteroylglutamates were found in cells grown in the absence of methionine. These levels were markedly reduced when L-methionine was supplemented in the culture medium. This reduction was greatest when *L. casei* was employed. The difference in the response of *L. casei* and *P. cerevisiae* given by the lower line (Figure 2) indicates that in the presence of methionine the levels of methylated derivatives were drastically reduced.

FIGURE 2

*THE EFFECT OF L-METHIONINE ON THE LEVELS OF PTEROYLGLUTAMATE DERIVATES*

Cells were cultured for 6 hours in basal media supplemented with various concentrations of L-methionine. Pteroylglutamates were then extracted in ascorbate and assayed with *L. casei* (o) and *P. cerevisiae* (▲). The data are in PteGlu equilavents for *L. casei* and 5-HCO-$H_4$PteGlu equilavents for *P. cerevisiae*. The dotted line is the difference in the response of *L. casei* and *P. cerevisiae*.


μg PTEROYLGLUTAMETE /g D. WT.
0
10
20
30
40
0
0.2
0.6
1.0
1.4
2.0
2.5
L-METHIONINE (μ moles /ml)

As yeast is known to contain a large proportion of highly conjugated pteroylglutamates (Schertel *et al.*, 1965), it was of interest to determine whether L-methionine would also reduce the level of these derivatives. To examine this, the extracts were treated with γ-glutamyl carboxypeptidases isolated from chicken pancreas (Mims and Laskowski, 1945) and 3-day-old pea cotyledons (Roos and Cossins, 1971). Activities of these enzymes were routinely checked (Table 3) using Difco-Bacto yeast extract as substrate (Roos and Cossins, 1971).

The levels of pteroylglutamates in the extracts after incubation with the pea cotyledon enzyme are shown in Figure 3. Treatment with this enzyme increased the levels of the derivatives by approximately 4-6 fold. As before, yeast grown in the absence of L-methionine contained the highest level of conjugated pteroylglutamate and reductions of this occurred when methionine was added to the culture media (Figure 3). It is of interest to note that, after pea cotyledon hydrolase treatment, both assay organisms gave approximately the same values for total pteroylglutamate content. This may indicate that the conjugated derivatives are largely formylated and unsubstituted derivatives of $H_4$PteGlu.

*Levels of Pteroylglutamate Derivatives during Growth and the Effect of L-methionine*

Yeast cultures, supplemented with L-methionine (2.5

TABLE 3. *HYDROLYSIS OF CONJUGATED PTEROYLGLUTAMATES PRESENT IN DIFCO-BACTO YEAST EXTRACT*

| Reaction system | Pteroylglutamate content as determined by *L. casei* (mμg) | |
|---|---|---|
| | Pea cotyledon enzyme | Chicken pancreas enzyme |
| Without enzyme | 208 | 182 |
| With enzyme | 2,440 | 1,940 |
| Pteroylglutamates released | 2,232 | 1,758 |

The extract was prepared by dissolving 20 g of Difco-Bacto yeast extract in 100 mls of 1% (w/v) potassium ascorbate (pH 6.0). The solution was heated at 95°C for 10 minutes, cooled rapidly and centrifuged at 18,000 x g for 20 minutes at 2°C. The solution was stored frozen until required. Reaction systems with the pea cotyledon enzyme contained 0.1 M sodium acetate buffer (pH 4.5), 1% (w/v) K ascorbate, 1 ml of yeast extract and 1 ml of enzyme solution in a final volume of 10 mls. For the chicken pancreas enzyme, the reaction systems contained 7 ml 0.2 M sodium borate buffer (pH 7.8), 1 ml of 0.1 M calcium chloride, 1 ml of yeast extract and 1 ml of enzyme solution in a final volume of 10 mls. The reaction systems were incubated at 35°C for 5 hours (chicken pancreas enzyme) and 3 hours (pea cotyledon enzyme). Reaction systems containing boiled enzyme failed to increase the pteroylglutamate content as assayed with *L. casei*. The data are in PteGlu equilavents.

FIGURE 3

*THE EFFECT OF L-METHIONINE ON THE LEVELS OF CONJUGATED PTEROYLGLUTAMATE DERIVATIVES*

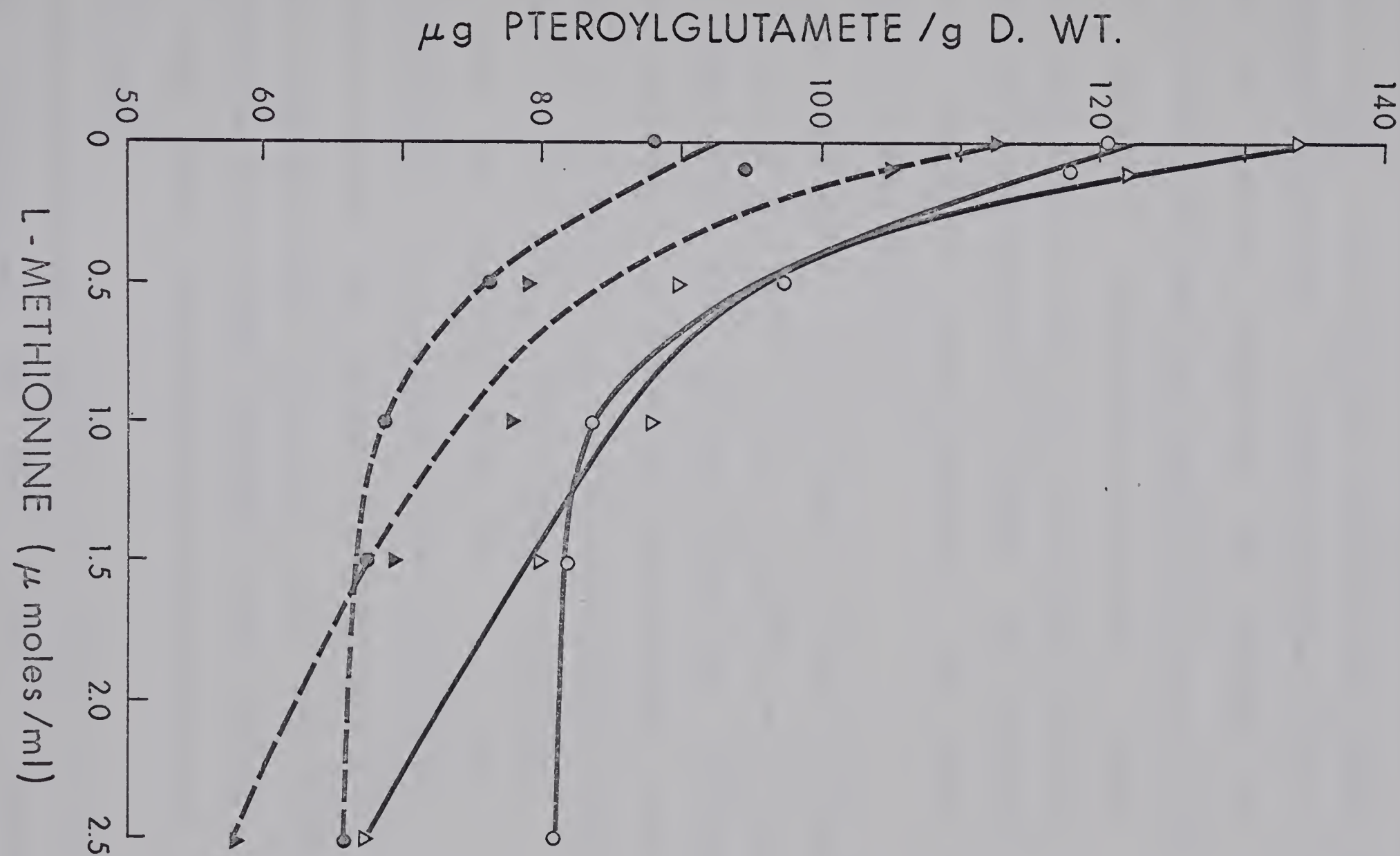
Cells were cultured for 6 hours in basal media supplemented with various concentrations of L-methionine. Pteroylglutamates were extracted in ascorbate. Levels of polyglutamyl derivatives were assayed microbiologically after incubation of the extracts with a pteroylpolyglutamate hydrolase isolated from pea cotyledons. The data are in PteGlu equilavents for *L. casei* and 5-HCO-$H_4$PteGlu equilavents for *P. cerevisiae*.

o——o total pteroylglutamates as assayed with *L. casei*

●---● conjugated pteroylglutamates as assayed with *L. casei*

Δ——Δ total pteroylglutamates as assayed with *P. cerevisiae*

▲---▲ conjugated pteroylglutamates as assayed with *P. cerevisiae*


μg PTEROYLGLUTAMETE /g D. WT.
50
60
80
100
120
140
0
0.5
1.0
1.5
2.0
2.5
L-METHIONINE (μ moles/ml)

μmole/ml), were harvested at different times during growth and ascorbate extracts were prepared as described earlier. At the end of 24 hours, the cells were harvested and washed with sterile demineralized water. A sample of these washed cells was removed for ascorbate extraction. The remainder were divided into equal portions and transferred to new culture media with and without L-methionine supplements. During this second growth period, the cells were again harvested at intervals and extracted with ascorbate. The resulting extracts were assayed for pteroylglutamates. The results are illustrated in Figure 4a and b.

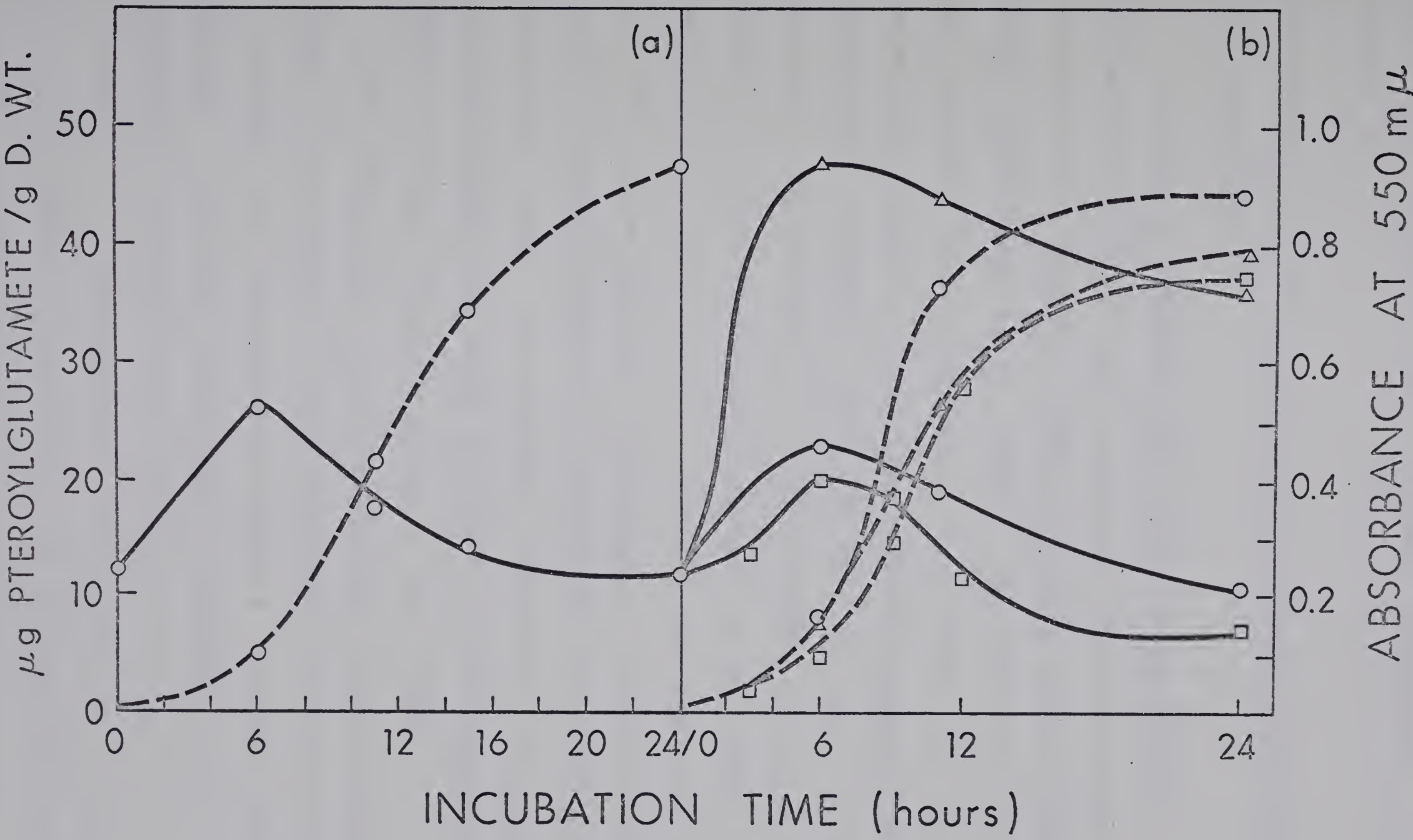
The pteroylglutamate levels of yeast cultures increased continuously during the first 6 hours of growth, at the end of which maximal levels were reached (Figure 4a). Following this, pteroylglutamate levels declined progressively to the values observed initially. During the second growth cycle, in different culture media, similar changes in pteroyl-glutamate levels were observed (Figure 4b). As in the earlier experiments, cells cultured in the presence of L-methionine contained lower levels of pteroylglutamates throughout the growth cycle. It is also clear from the data that L-methionine gave greater decreases in the pteroyl-glutamate levels of the cells when they were in the post-exponential phase.

When the extracts were incubated with chicken pancreas γ-glutamyl carboxypeptidase, the levels of pteroylglutamates were increased by 3-4 fold, however, the methionine-grown

FIGURE 4

*CHANGES IN PTEROYLGLUTAMATE POOL SIZE DURING GROWTH*

Cells were cultures in basal medium with a supplement of 2.5 µmoles/ml of L-methionine (Figure 4a). Absorbance at 550 mµ (dotted lines) and pteroylglutamate levels (solid lines) were determined at the times indicated. At the end of 24 hours, the cells were harvested and transferred to the fresh media (Figure 4b) without methionine supplement (Δ); and with L-methionine supplements of 2.5 µmole/ml (o) and 5 µmoles/ml (□) respectively. Absorbance at 550 mµ and pteroylglutamate levels were determined as indicated during this 2nd growth cycle (Figure 4b). The data are in PteGlu equilavents as determined by *L. casei*.


(a)
(b)
μg PTEROYLGLUTAMETE /g D. WT.
ABSORBANCE AT 550 mμ
0
10
20
30
40
50
0.2
0.4
0.6
0.8
1.0
0
6
12
16
20
24/0
6
12
24
INCUBATION TIME (hours)

cells still contained lower levels of conjugated derivatives (Figure 5).

*Chromatography of Pteroylglutamate Derivatives Extracted from Yeast Cells*

In order to determine the nature of the pteroylglutamates present in yeast, extracts of 24 hour yeast cultures were subjected to DEAE-cellulose column chromatography (Sotobayashi *et al.*, 1966). The collected fractions were assayed with *L. casei* and *P. cerevisiae*. For reference, the elution pattern of individual pteroylglutamates was established. Authentic samples (0.05-0.10 µg) of 10-HCO-$H_4$PteGlu, 5-HCO-$H_4$PteGlu, 5-$^{14}CH_3$-$H_4$PteGlu, $H_4$PteGlu and PteGlu-2-$^{14}C$ were individually applied to the columns and the fractions collected were assayed microbiologically as described in the Materials and Methods. For such standardization, 10-HCO-$H_4$PteGlu was synthesized from 5-HCO-$H_4$PteGlu (Rabinowitz, 1963). The positions of elution of authentic pteroylglutamates are shown in Table 4. The results are similar to those obtained earlier by Roos (1971).

The differential assay of individual pteroylglutamates present in an extract of yeast cultured in the basal medium for 24 hours, is illustrated in Figure 6. Several peaks were separated. These were identified by reference to (a) differential microbiological response to *L. casei* and *P. cerevisiae*; (b) a comparison of the position of elution with that of the authentic derivatives and (c) the effect of pea cotyledon hydrolase treatment of individual fractions.

FIGURE 5

*CHANGES IN PTEROYLPOLYGLUTAMATE POOL SIZE DURING GROWTH*

Levels of pteroylpolyglutamates were assayed microbiologically after treatment with chicken pancreas γ-glutamyl carboxypeptidase. The extracts were those assayed in the experiment shown in Figure 4. The data are in PteGlu equilavents as determined by *L. casei*. The levels of pteroylglutamate derivatives in the various cultures are:

o---o conjugated pteroylglutamates in cells grown in the presence of L-methionine (2.5 μmoles/ml)

Δ---Δ conjugated pteroylglutamates in cells grown in the absence of L-methionine

o——o total pteroylglutamates in cells grown in the presence of L-methionine (2.5 μmoles/ml)

Δ——Δ total pteroylglutamates in cells grown in the absence of L-methionine

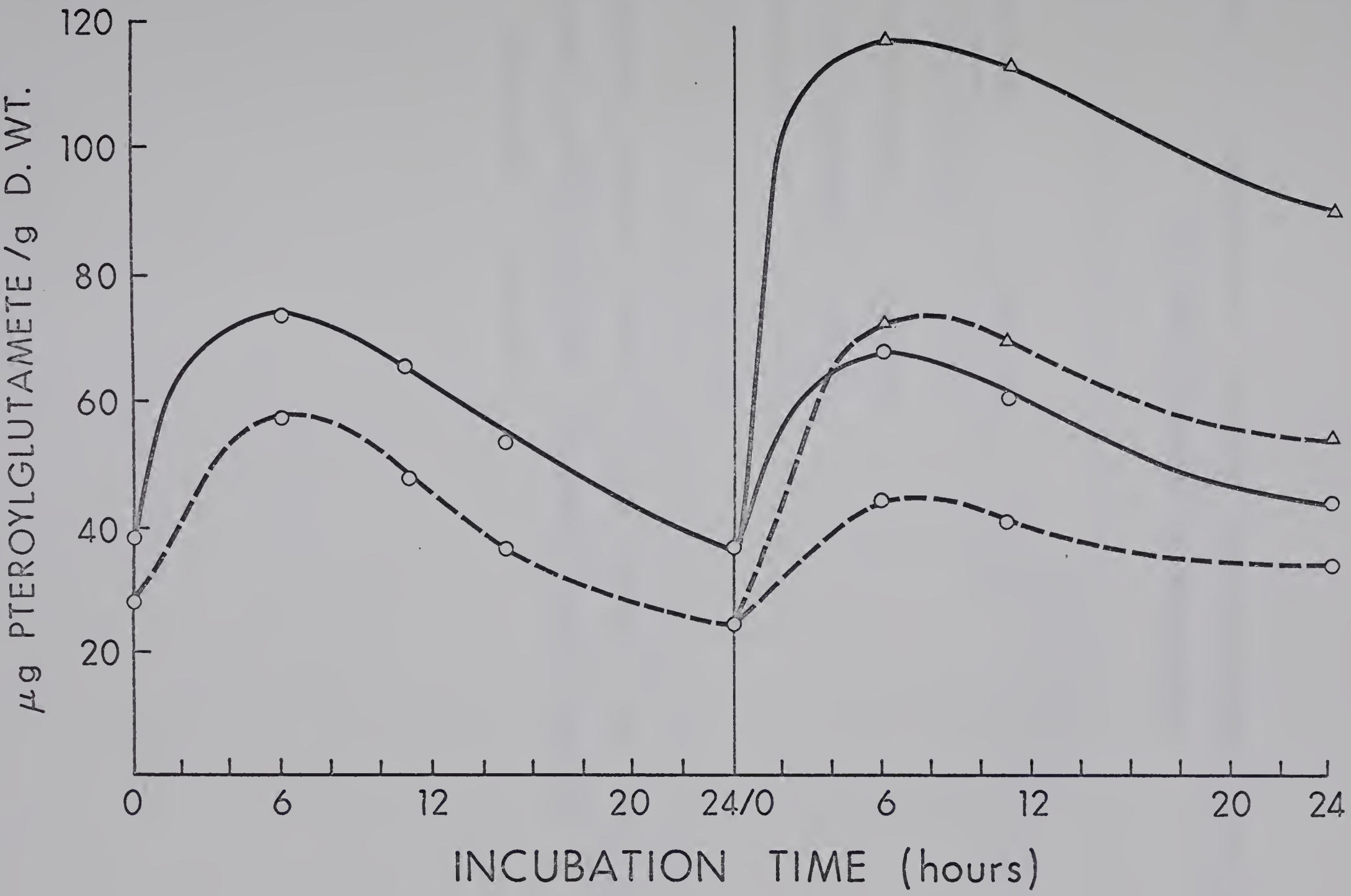

120
100
80
60
40
20
µg PTEROYLGLUTAMETE /g D. WT.
0
6
12
20
24/0
6
12
20
24
INCUBATION TIME (hours)

TABLE 4. *SEPARATION OF AUTHENTIC PTEROYLGLUTAMATE DERIVATIVES BY DEAE-CELLULOSE CHROMATOGRAPHY*

| Compound | Fractions | Growth Response | |
|---|---|---|---|
| | | *L. casei* | *P. cerevisiae* |
| 10-HCO-$H_4$PteGlu | 34-47 | + | + |
| 5-HCO-$H_4$PteGlu | 58-70 | + | + |
| 5-$^{14}CH_3$-$H_4$PteGlu | 65-75 | + | — |
| $H_4$PteGlu | 70-79 | + | + |
| PteGlu-2-$^{14}C$ | 125-145 | + | — |

Authentic samples (0.05-0.10 µg or 0.01 µCi) were chromatographed on DEAE-cellulose, followed by assay employing *L. casei* and *P. cerevisiae*.

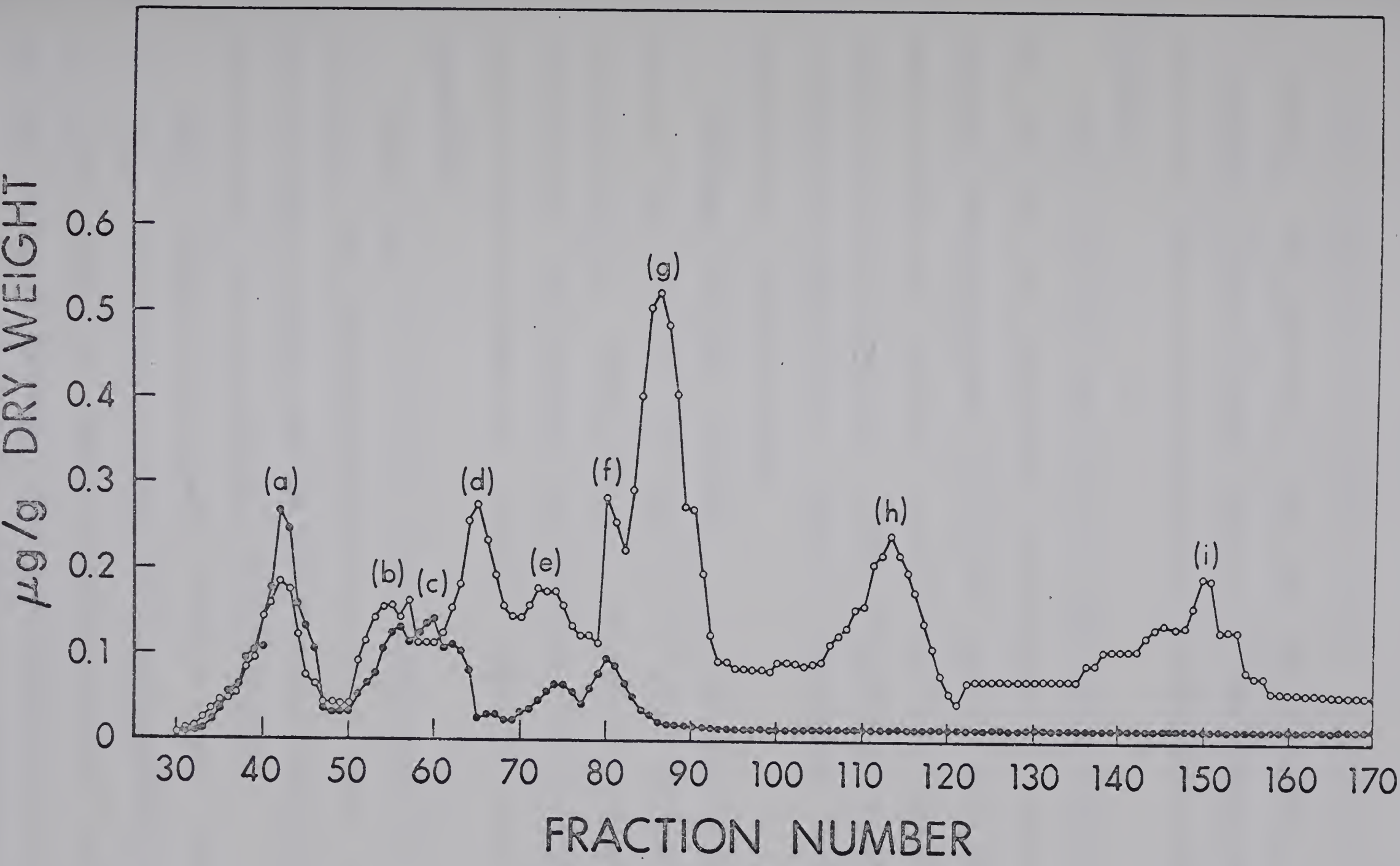
FIGURE 6

*CHROMATOGRAPHY AND DIFFERENTIAL ASSAY OF PTEROYLGLUTAMATES FROM YEAST GROWN FOR 24 HOURS IN THE ABSENCE OF METHIONINE*

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *L. casei* (o) and *P. cerevisiae* (●). The peaks are identified as:

(*a*), 10-HCO-$H_4$PteGlu; (*b*), 10-HCO-$H_4$PteGlu$_2$;
(*c*), 5-HCO-$H_4$PteGlu; (*d*), 5-$CH_3$-$H_4$PteGlu;
(*e*), $H_4$PteGlu; (*f*), 5-HCO-$H_4$PteGlu$_2$; (*g*), 5-$CH_3$-$H_5$PteGlu$_2$;
(*h*), 5-$CH_3$-$H_4$PteGlu$_3$; (*i*), unidentified conjugated derivatives.

No growth response was obtained before fraction 30 or after fraction 160. The data are in PteGlu equilavents for *L. casei* and 5-HCO-$H_4$PteGlu equilavents for *P. cerevisiae*.


µg/g DRY WEIGHT
0.6
0.5
0.4
0.3
0.2
0.1
0
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
FRACTION NUMBER

Peak *a* supported the growth of both *L. casei* and *P. cerevisiae*. The position of this peak corresponded to that of authentic 10-HCO-$H_4$PteGlu (Table 4). Confirmation was obtained as Peak *a* chromatographed with authentic 10-HCO-$H_4$PteGlu.

Peak *b* supported the growth of both assay organisms. This peak does not correspond in position to any of the authentic pteroylglutamate derivatives used in this study. However, this peak occurred in a position (fractions 50-56) similar to that of authentic 10-HCO-$H_4$PteGlu$_2$ as reported by Cossins and Shah (1971). Peak *b* is, therefore, tentatively identified as 10-HCO-$H_4$PteGlu$_2$.

Although not shown in Figure 6, an additional peak (peak *c*, Figure 6) was often encountered after chromatography of yeast extracts. On the basis of the general criteria cited earlier, this peak was identified as 5-HCO-$H_4$PteGlu. Levels of this compound were routinely measured using *P. cerevisiae*.

Peak *d* gave no detectable growth response with *P. cerevisiae*, and chromatographed with 5-$CH_3$-$H_4$PteGlu. On the basis of these findings and the other criteria mentioned earlier, peak *d* is identified as 5-$CH_3$-$H_4$PteGlu.

Peak *e* supported the growth of both *L. casei* and *P. cerevisiae*. The position of this peak corresponded to that of $H_4$PteGlu (Table 4). When co-chromatography was carried out with authentic $H_4$PteGlu, this peak increased in size and by the theoretical amount. Peak *e* is, therefore,

identified as $H_4$PteGlu.

As peak *f* supported the growth of both assay organisms, and was located at a position corresponding to that of authentic 5-HCO-$H_4$PteGlu$_2$ (Roos, 1971), it was tentatively identified as 5-HCO-$H_4$PteGlu$_2$.

Peaks *g* - *i* supported only the growth of *L. casei*. They might, therefore, contain either (a) triglutamyl forms of formylated or unsubstituted derivatives or (b) di- and triglutamyl forms of methyl derivatives. The first possibility was unlikely as differential assays of these compounds after hydrolase treatment of the separated peaks, revealed that Peaks *g* and *h*, although supporting the growth of *L. casei*, still failed to support the growth of *P. cerevisiae*. These peaks also occurred at positions very similar to those of 5-$CH_3$-$H_4$PteGlu$_2$ and 5-$CH_3$-$H_4$PteGlu$_3$ (Cossins and Shah, 1971). Therefore, Peak *g* is tentatively designated as 5-$CH_3$-$H_4$PteGlu$_2$ and Peak *h* as 5-$CH_3$-$H_4$PteGlu$_3$. Peak *i*, after such enzyme treatment again supported the growth of *L. casei* but now gave a slight growth response with *P. cerevisiae* (20% that of *L. casei*). Furthermore, this peak disappeared when the whole extract was hydrolase-treated before chromatography. Peak *i* thus appears to contain more than one polyglutamyl derivative.

In these studies, no evidence was obtained for the occurrence of $H_2$PteGlu, PteGlu, 5,10-$CH_2$=$H_4$PteGlu; 5,10-CH≡$H_4$PteGlu and 5-HCNH-$H_4$PteGlu, although these compounds have been implicated in one-carbon metabolism (Blakley, 1969).

This does not, however, dispute their natural occurrence in yeast, especially as 5,10-$CH_2$=$H_4$PteGlu and 5,10-CH≡$H_4$PteGlu and 5-HCNH-$H_4$PteGlu are unstable under the present conditions of extraction and isolation.

*Effects of Methionine on Individual Pteroylglutamate Derivatives*

The preliminary experiments described above showed that the levels of pteroylglutamate derivatives in yeast were drastically reduced in the presence of an exogenous supply of methionine (Figures 2, 3, 4 and 5). These reductions most likely involved the majority of the derivatives present in the pteroylglutamate pool. This likelihood was confirmed when such extracts, prepared from 24 hour-old yeast before and after hydrolase treatment, were subjected to column chromatography. The results are illustrated in Figures 7 and 8 and Table 5.

Before hydrolase treatment, very low levels of all principal derivatives were found in the methionine-grown cells (Figure 7). When compared with the derivatives present in cells grown on the basal medium, it is clear that the levels of methyl derivatives (Peaks *d*, *g* and *h*) were drastically reduced by the presence of methionine. The reduction in the levels of these derivatives were as great as 82% to 95% (Table 5). Derivatives eluted in peak *i* in the absence of methionine in the medium were completely absent in the methionine-grown cells (Figure 7, Table 5). In contrast,

FIGURE 7

*CHROMATOGRAPHY OF PTEROYLGLUTAMATES FROM YEAST AFTER 24 HOURS OF GROWTH*

Derivatives isolated from the cells grown for 24 hours in the basal medium without methionine supplement (o) and in the medium containing 2.5 µmoles/ml L-methionine (●) were separated by DEAE-cellulose chromatography followed by assay using *L. casei*. The peaks are identified as:

(*a*), 10-HCO-$H_4$PteGlu; (*b*), 10-HCO-$H_4$PteGlu$_2$; (*c*), 5-HCO-$H_4$PteGlu; (*d*), 5-$CH_3$-$H_4$PteGlu; (*e*), $H_4$PteGlu; (*f*), 5-HCO-$H_4$PteGlu$_2$; (*g*), 5-$CH_3$-$H_4$PteGlu$_2$; (*h*), 5-$CH_3$-$H_4$PteGlu$_3$; (*i*), unidentified conjugated derivatives.

No growth response was obtained before fraction 30 or after fraction 160. The data are in PteGlu equilavents for *L. casei*.

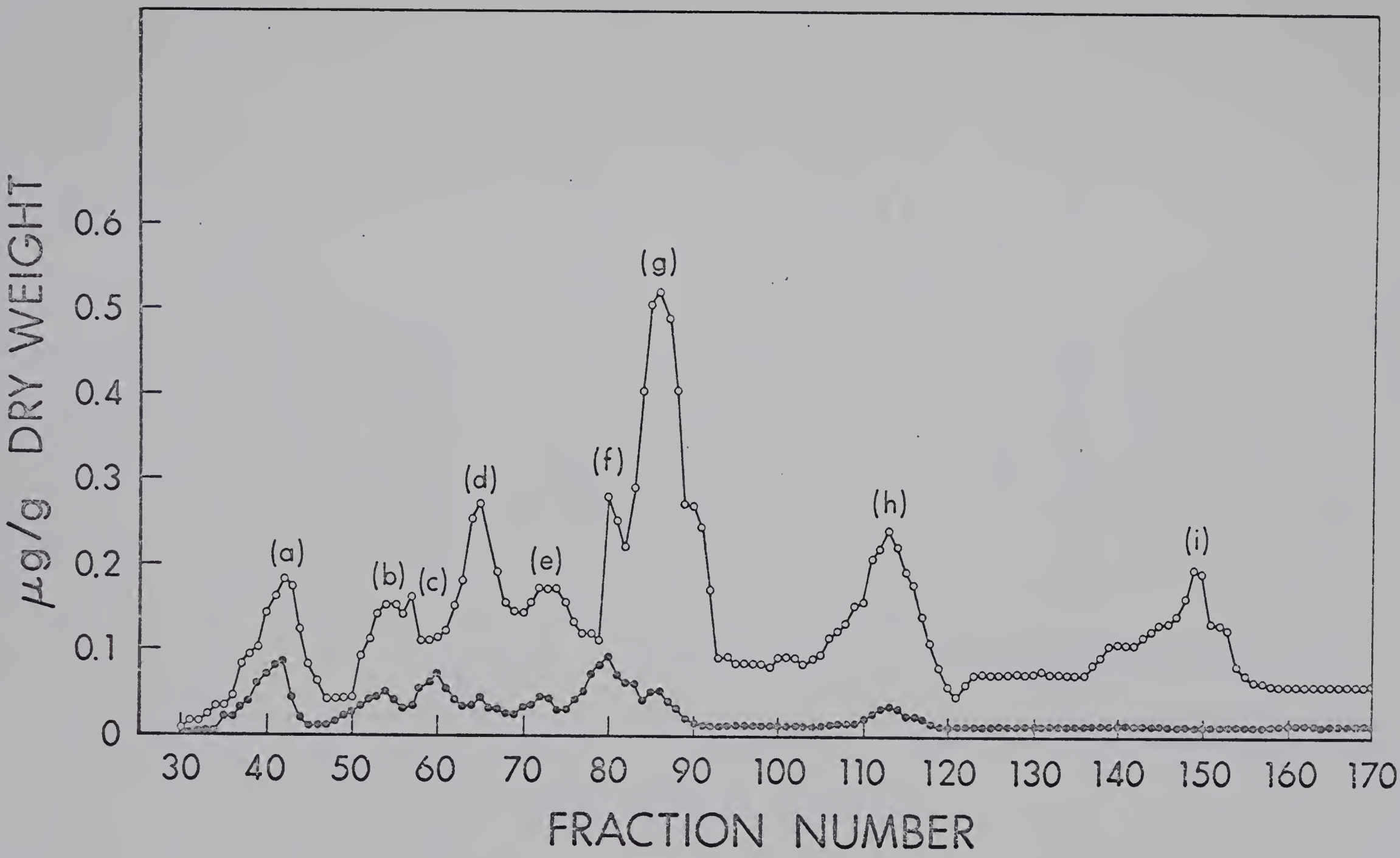

µg/g DRY WEIGHT
0.6
0.5
0.4
0.3
0.2
0.1
0
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
FRACTION NUMBER

FIGURE 8

*CHROMATOGRAPHY OF HYDROLASE-TREATED EXTRACTS OBTAINED FROM CELLS GROWN FOR 24 HOURS*

Extracts of cells grown for 24 hours in the basal medium without methionine supplement (o) and in the medium containing 2.5 μmoles/ml L-methionine (●) were treated with pea cotyledon pteroylpolyglutamate hydrolase. Pteroylglutamates released by hydrolase treatment were then subjected to chromatography on DEAE-cellulose. Peaks are identified as:

(*a*), 10-HCO-$H_4$PteGlu; (*b*), 10-HCO-$H_4$PteGlu$_2$;

(*c*), 5-HCO-$H_4$PteGlu; (*f*), 5-HCO-$H_4$PteGlu$_2$;

(*g*), 5-$CH_3$-$H_4$PteGlu$_2$; (*h*), conjugated derivatives.

The data are in PteGlu equilavents for *L. casei*.

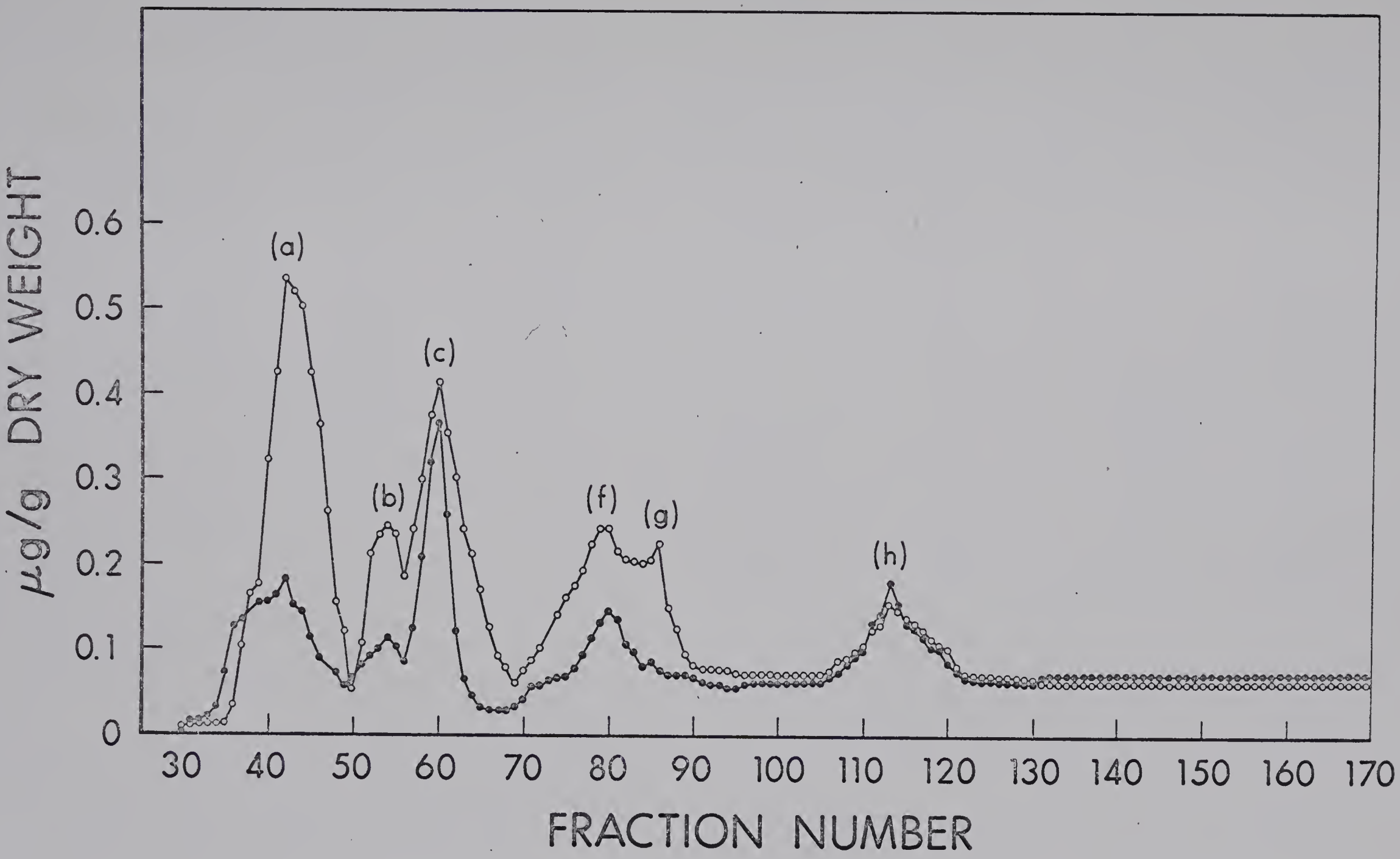

µg/g DRY WEIGHT
0.6
0.5
0.4
0.3
0.2
0.1
0
(a)
(b)
(c)
(f)
(g)
(h)
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
FRACTION NUMBER

TABLE 5

Data illustrating distribution of pteroylglutamate derivatives following DEAE-cellulose column chromatography of yeast extract prepared in ascorbate (pH 6.0). Hydrolysis of conjugated derivatives was achieved by incubation of the extracts with pea cotyledon hydrolase. The data are in PteGlu equilavents as determined by *L. casei*. In all cases, recoveries of pteroylglutamates were 95 ± 10%.

TABLE 5. *LEVELS OF INDIVIDUAL PTEROYLGLUTAMATE DERIVATIVES IN 24 HOUR-OLD YEAST CULTURED IN THE PRESENCE AND IN ABSENCE OF L-METHIONINE* (2.5 µmole/ml)

| Derivatives ( g/g.d.wt.) | With Methionine (2.5mM) | | Without Methionine | | % reduced by Methionine | |
|---|---|---|---|---|---|---|
| | Before Hydrolysis | After Hydrolysis | Before Hydrolysis | After Hydrolysis | Before Hydrolysis | After Hydrolysis |
| 10-HCO-$H_4$PteGlu | 1.52 | 13.70 | 3.39 | 39.24 | 60.0 | 69.0 |
| 10-HCO-$H_4$PteGlu$_2$ | 2.18 | 4.08 | 2.07 | 15.22 | 8.0 | 76.3 |
| 5-HCO-$H_4$PteGlu | 1.86 | 14.96 | 2.27 | 22.19 | 18.0 | 40.0 |
| 5-$CH_3$-$H_4$PteGlu | 0.67 | -- | 3.32 | -- | 82.0 | -- |
| $H_4$PteGlu | 1.52 | -- | 2.83 | -- | 53.0 | -- |
| 5-HCO-$H_4$PteGlu$_2$ | 2.22 | 5.98 | 2.75 | 14.34 | 29.0 | 63.0 |
| 5-$CH_3$-$H_4$PteGlu$_2$ | 0.54 | -- | 9.19 | 6.69 | 95.0 | -- |
| Peak *h* | 0.96* | 8.21** | 5.89* | 10.64** | 86.0 | 31.7 |
| Unidentified conjugated derivatives (peak *i*) | *n.d.* | -- | 3.19 | -- | 100.0 | -- |
| Total | 11.06 | 46.94 | 32.63 | 108.31 | 64.0 | 61.7 |

*n.d.* - no detectable growth response with *L. casei*.

* tentatively designated as 5-CH -H PteGlu ; ** contained compounds capable of supporting *L. casei* and *P. cerevisiae*.

smaller decreases ranging from 8% to 60% were observed in the levels of 10-HCO-$H_4$PteGlu, 10-HCO-$H_4$PteGlu$_2$, 5-HCO-$H_4$PteGlu, 5-HCO-$H_4$PteGlu$_2$ and $H_4$PteGlu when this amino acid was supplied in the medium (Figure 7, Table 5).

Pea cotyledon hydrolase treatment of the extracts from these cells before chromatography resulted in large increases in the levels of formyl derivatives (Figure 8, Table 5) but did not appreciably change the levels of methyl derivatives. In the methionine-grown cells the formylated compounds which occurred as polyglutamyl derivatives were greatly decreased in amounts. Under these conditions, slightly smaller decreases also occurred in the levels of 5-HCO-$H_4$Pte Glu and Peak *h* (Figure 8) which in this case contained compounds capable of supporting *P. cerevisiae*.

Levels of pteroylglutamates in 6-hour-old cells before and after hydrolase treatment are shown in Table 6. A similar effect of methionine was observed. The levels of methyl and formyl derivatives before hydrolysis were greatly reduced by methionine feeding. Conjugated formyl derivatives produced by hydrolase treatment were again reduced in cells cultured in the presence of methionine (Table 6).

*Studies Involving Cell-free Extracts*

The results of previous experiments indicate that the decreases in pteroylglutamate pool associated with methionine feeding could be due to either greater utilization of pteroyl-glutamates or a decreased production of these derivatives in the presence of exogenous methionine. To test these

TABLE 6

Data illustrating distribution of pteroylglutamate derivatives following DEAE-cellulose column chromatography of yeast prepared in ascorbate (pH 6.0). Hydrolysis of conjugated derivatives was achieved by incubation of the extracts with pea cotyledon hydrolase. The data are in PteGlu equilavents as determined by *L. casei*. In all cases, the recovery of pteroylglutamates was 95 ± 10%.

TABLE 6. *LEVELS OF INDIVIDUAL PTEROYLGLUTAMATE DERIVATIVES IN 6-HOUR-OLD YEAST CULTURED IN THE PRESENCE AND ABSENCE OF L-METHIONINE* (2.5 µmole/ml)

| Derivatives (µg/g.d.wt.) | With methionine (2.5 µmole/ml) | | Without methionine | | % reduction by methionine | |
|---|---|---|---|---|---|---|
| | Before Hydrolysis | After Hydrolysis | Before Hydrolysis | After Hydrolysis | Before Hydrolysis | After Hydrolysis |
| 10-HCO-$H_4$PteGlu | 3.51 | 24.65 | 4.48 | 32.97 | 22.3 | 24.3 |
| 10-HCO-$H_4$PteGlu$_2$ | 3.20 | 15.09 | 4.63 | 19.43 | 30.4 | 22.2 |
| 5-HCO-$H_4$PteGlu | 1.98 | 21.34 | 2.70 | 31.09 | 26.7 | 31.6 |
| 5-$CH_3$-$H_4$PteGlu | 0.51 | -- | 4.85 | -- | 89.5 | -- |
| $H_4$PteGlu | 1.47 | 5.45 | 1.75 | 9.71 | 16.7 | 43.3 |
| 5-HCO-$H_4$PteGlu$_2$ (Peak *f*) | 2.49 | 11.65 | 3.42 | 21.35 | 27.2 | 45.5 |
| 5-$CH_3$-$H_4$PteGlu$_2$ (Peak *g*) | 0.33 | -- | 8.57 | -- | 96.2 | -- |
| Peak *h* | 1.21* | 3.43** | 5.30* | 4.45** | 81.2 | 24.4 |
| Peak *i* | 0.99 | -- | 4.77 | -- | 79.2 | -- |
| Total | 15.49 | 81.60 | 40.48 | 119.00 | 61.8 | 32.4 |

* Tentatively designated as 5-$CH_3$-$H_4$PteGlu$_3$; ** contained compounds capable of supporting *L. casei* and *P. cerevisiae*.

possibilities, levels of the key enzymes involved in one-carbon metabolism were examined using the cell-free extracts prepared from cells grown in different media. The levels of these enzymes are shown in Table 7. The effects of methionine on these enzyme activities were also studied in other experiments.

(a) 5,10-$CH_2$=$H_4$PteGlu reductase

Repression of this enzyme by high concentrations of methionine in a methionine-$B_{12}$ auxotroph of *E. coli* is well documented (Dickerman and Weissbach, 1964; Taylor *et al.*, 1966). In the present investigation, repression of this enzyme by methionine was not observed (Table 7). The enzyme was, however, inhibited by methionine when this amino acid was included in the reaction system (Figure 9). Combepine *et al.* (1971) obtained similar results for this enzyme isolated from yeast cultured in a minimal medium.

(b) 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase

Burton *et al.* (1968) have reported that this transmethylase in *Saccharomyces cerevisiae* utilizes 5-$CH_3$-$H_4$Pte$Glu_2$ or 5-$CH_3$-$H_4$PteGlu$_3$ to form methionine. However, in their studies, some enzyme activity was measurable when 5-$CH_3$-$H_4$PteGlu was used as the methyl donor. In the present studies, 5-$^{14}CH_3$-$H_4$PteGlu was used as substrate to examine possible effects of methionine on this enzyme.

Enzyme activity was measured by the isotopic method as described in Materials and Methods. Assays of this enzyme showed that when the cells were grown in the presence of

TABLE 7. *EFFECT OF L-METHIONINE ON THE LEVELS OF PTEROYL-GLUTAMATE ENZYMES*

| Enzyme | With methionine supplement (2.5 µmole/ml) | | Without methionine supplement | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| 10-HCO-$H_4$PteGlu synthetase* | 0.28 | 0.44 | 0.32 | 0.46 |
| Serine hydroxymethyl-transferase** | 5,200 | 4,200 | 5,100 | 3,900 |
| 5,10-$CH_2$=$H_4$PteGlu*** reductase | 100 | 73 | 115 | 80 |
| 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase**** | 15.7 | 11.9 | 43.2 | 38.5 |

* Product formed µmole/mg protein/hr.
** Product formed cpm/mg protein/hr.
*** Product formed µµmole/mg protein/10 min.
**** Product formed µµmole/mg/hr.

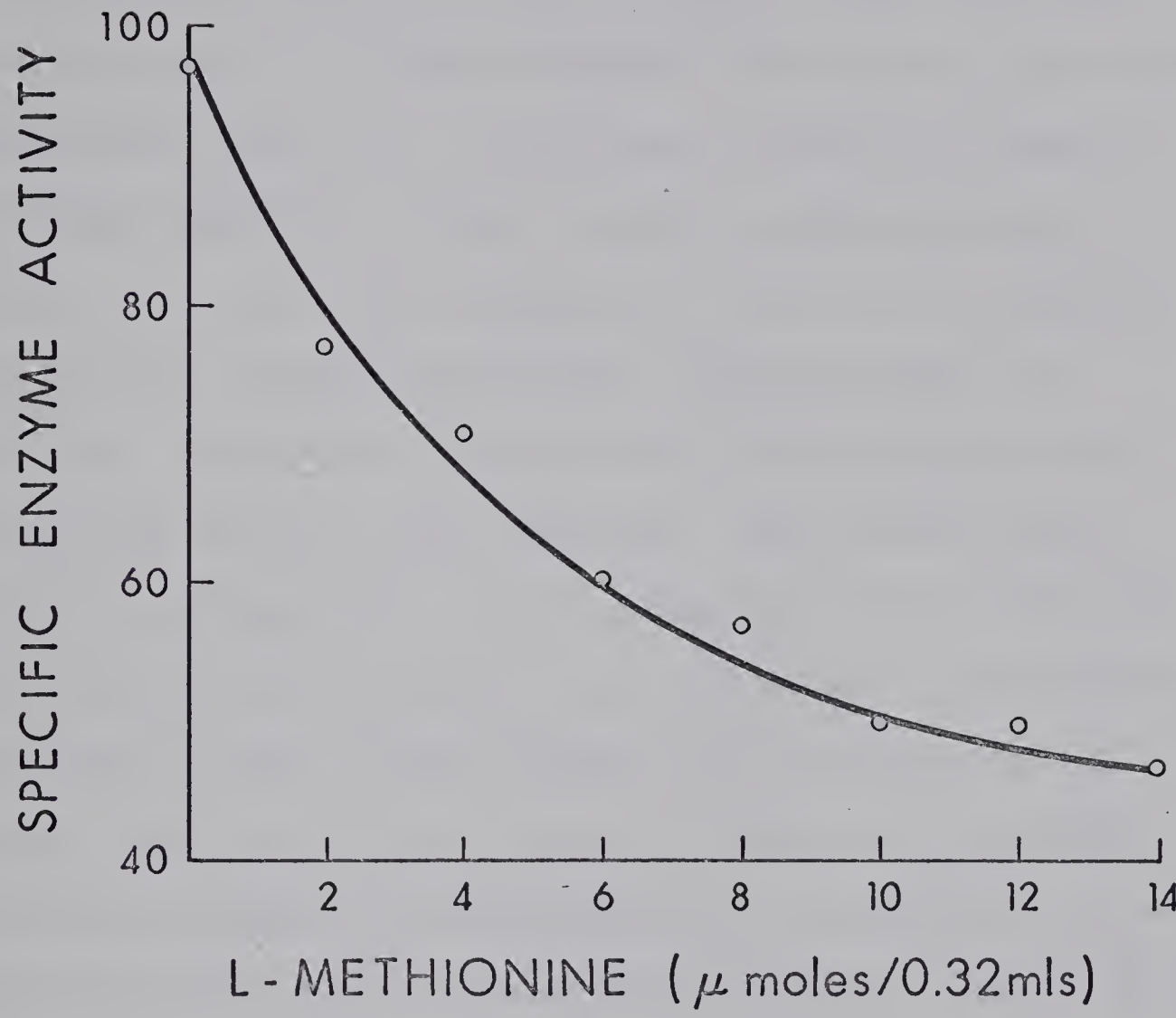
The activities of the enzymes indicated were measured as described in the Materials and Methods. Cell-free extracts of yeast cells grown in the presence and absence of L-methionine (2.5 µmole/ml) were used as a source of enzyme. All assays were carried out in duplicate and the values obtained are averages of duplicate assays.

FIGURE 9

*THE EFFECT OF DIFFERENT CONCENTRATIONS OF L-METHIONINE ON THE ACTIVITY OF YEAST 5,10-$CH_2$=$H_4$PTEGLU REDUCTASE*

5,10-$CH_2$=$H_4$PteGlu reductase activity was measured using 5-$^{14}CH_3$-$H_4$PteGlu as described in Materials and Methods. Cell-free extracts of yeast cells grown in the absence of L-methionine were used as a source of enzyme.

All assays were carried out in duplicate and values obtained are averages of 2 separate experiments. Specific enzyme activity is expressed as μμmoles $^{14}$C-formaldehyde formed per mg protein in 10 minutes at 30°C.


SPECIFIC ENZYME ACTIVITY
100
80
60
40
2
4
6
8
10
12
14
L - METHIONINE ( μ moles/0.32mls)

methionine, enzyme activity was only 35% of that observed in yeast grown in the absence of this amino acid (Table 7). No inhibition of enzyme activity was observed when various concentrations of methionine were added to the reaction system containing the enzyme prepared from cells grown without methionine (Table 8). From these results, it may be assumed that synthesis of this enzyme is repressed by methionine. To test this assumption, cells were grown in the basal media containing various concentrations of L-methionine. Cell-free extracts were then prepared and transmethylase activity was measured. The results are illustrated in Figure 10. It is clear that the levels of this enzyme were decreased as the concentration of methionine supplemented in the culture medium was increased to 1.5 μmoles/ml. When cell-free extracts, prepared from cells grown in the presence of methionine (2.5 μmole/ml), where dialyzed overnight against potassium phosphate buffer (0.1 M, pH 6.9) no change in specific enzyme activity was observed.

(c) 10-HCO-$H_4$PteGlu synthetase

Because relatively low levels of formylated derivatives were observed when cells were cultured with a methionine supplement, it was of interest to determine whether these culture conditions affected the levels of 10-HCO-$H_4$PteGlu synthetase. Although small differences were observed in the specific activity of this enzyme (Table 7), they were on the whole too small to indicate enzyme repression. When various levels of L-methionine were included in the reaction system

TABLE 8. *EFFECT OF L-METHIONINE ON 5-$CH_3$-$H_4$PTEGLU:HOMOCYSTEINE TRANSMETHYLASE*

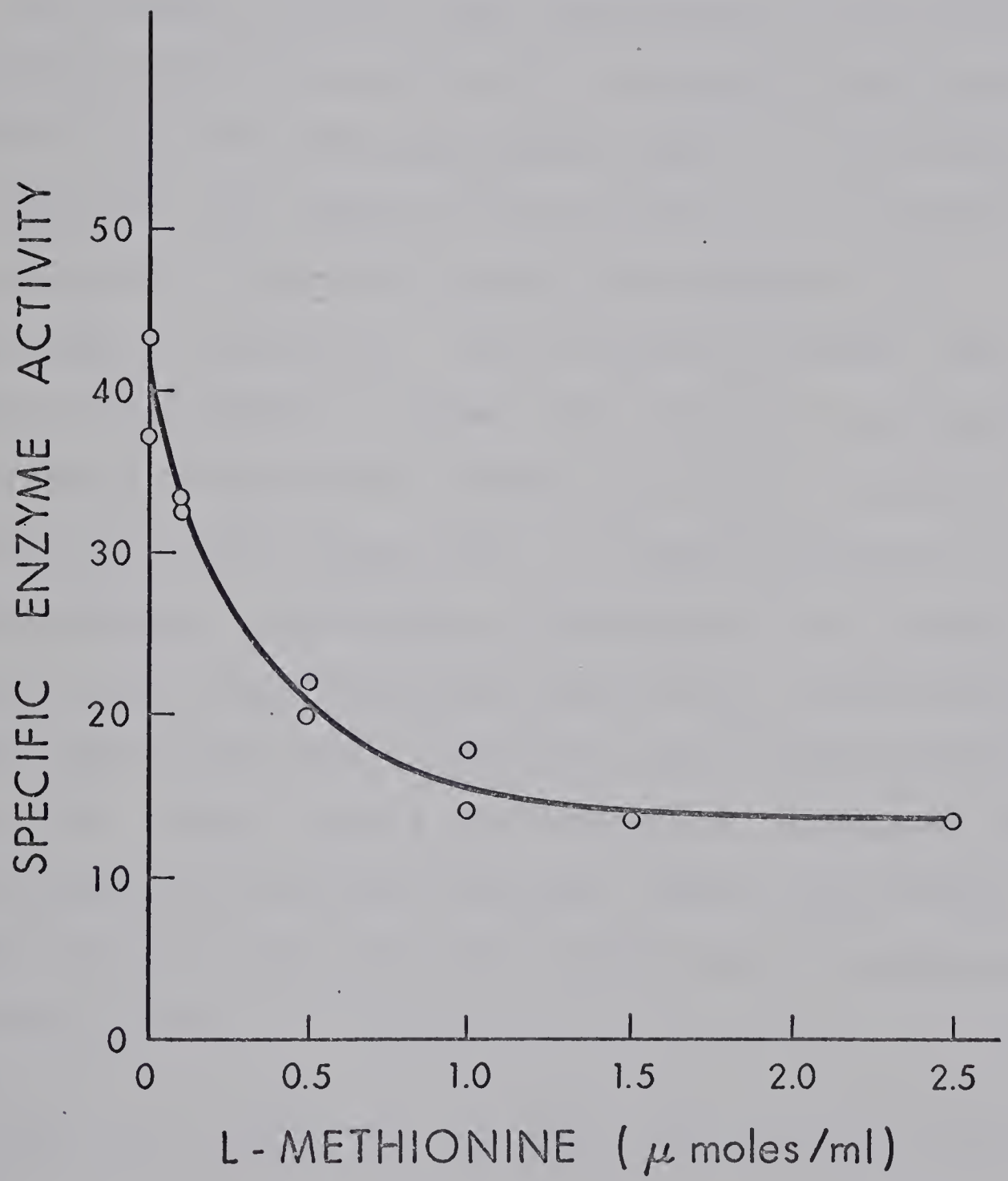
| L-methionine (µmoles) added to reaction system | Specific enzyme activity | |
|---|---|---|
| | Expt. 1 | Expt. 2 |
| 0 | 43.2 | 38.5 |
| 0.5 | 41.0 | 39.0 |
| 1.0 | 49.0 | 39.0 |
| 2.5 | 41.8 | 40.6 |
| 5.0 | 39.6 | 36.2 |
| 10.0 | 37.6 | 40.0 |
| 20.0 | 37.3 | 39.3 |

5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase activity was measured using 5-$^{14}CH_3$-$H_4$PteGlu as described in the Materials and Methods. Cell-free extracts of yeast cells grown in the absence of L-methionine were used as a source of enzyme. All assays were carried out in duplicate and values obtained are averages of duplicate assays. Specific enzyme activity is expressed as µµmoles methionine formed/mg protein/hr. at 30°C.

FIGURE 10

*REPRESSION OF 5-$CH_3$-$H_4$PTEGLU HOMOCYSTEINE TRANSMETHYLASE AS A FUNCTION OF L-METHIONINE CONCENTRATION*

Cells were grown for 6 hours in the basal culture medium supplemented with various concentrations of L-methionine. Cell-free extracts were assayed for enzyme activity by the standard isotope method using 5-$^{14}CH_3$-$H_4$PteGlu as described in the Materials and Methods section. Specific activity of enzyme is expressed as μμmole methionine formed/mg protein/ hour at 30°C.


SPECIFIC ENZYME ACTIVITY
50
40
30
20
10
0
0
0.5
1.0
1.5
2.0
2.5
L - METHIONINE ( μ moles /ml)

containing the enzyme prepared from cells grown in the absence of methionine, a decrease of approximately 20-30% was observed at relatively high concentrations of methionine (Table 9).

(d) Serine hydroxymethyltransferase

This enzyme catalyzes the interconversion of glycine and serine and is responsible for entrance of the β-carbon of serine into the pteroylglutamate pool. As reviewed in the Introduction, this enzyme has been shown to be involved in the synthesis of the methyl group of methionine in *Saccharomyces cerevisiae*. In the present studies, the assay of this enzyme (Table 7) shows that there was no significant difference in the specific enzyme activity of extracts prepared from cells grown with or without methionine in the culture medium. Furthermore, inhibition of this enzyme by methionine was not observed when various concentrations of this amino acid were included in the reaction system (Table 10). These results are, therefore, different from those reported by Bosford and Parks (1969) which showed that in this organism the enzyme was inhibited by L-methionine and SAM *in vitro*.

*The Effect of L-methionine on Levels of Free Amino Acids in Yeast*

In the present investigation, most of the key amino acids except methionine were supplied in the culture media. In order to demonstrate possible regulation of methyl group biogenesis from the pteroylglutamate pool such cultures were

TABLE 9. *EFFECT OF L-METHIONINE ON 10-HCO-$H_4$PTEGLU SYNTHETASE ACTIVITY*

| L-methionine (µmoles) in the reaction system | Specific enzyme activity (µmole/mg protein/hr | |
|---|---|---|
| | Expt. 1 | Expt. 2 |
| 0 | 0.32 | 0.46 |
| 2.5 | 0.28 | 0.42 |
| 5.0 | 0.28 | 0.34 |
| 10.0 | 0.28 | 0.38 |
| 15.00 | 0.24 | 0.32 |
| 20.0 | 0.25 | 0.29 |

Extracts were prepared from cells grown in the absence of L-methionine for 6 hours. The reaction system contained 100 µmoles triethanolamine buffer (pH 8.0), 150 µmoles Tris formate (pH 8.0), 2.5 µmoles $MgCl_2$, 200 µmoles KCl, 4 µmoles DL-$H_4$PteGlu, 2 µmoles ATP, 0.1 ml cell-free extract and the inclusion of various amounts of L-methionine in a total volume of 1 ml. The reaction mixture was incubated at 30°C for 10 minutes. Specific activity is defined as µmoles of product/mg protein/hr.

TABLE 10. *EFFECT OF L-METHIONINE ON SERINE HYDROXYMETHYL-TRANSFERASE ACTIVITY*

| L-methionine (μmoles) added to the reaction system | Specific enzyme activity (cpm/mg protein/hr) | |
|---|---|---|
| | Expt. 1 | Expt. 2 |
| 0 | 5,100 | 3,900 |
| 5 | 5,250 | 3,570 |
| 10 | 6,400 | 4,460 |
| 20 | 6,320 | 4,600 |
| 30 | 6,230 | 4,710 |
| 40 | 6,370 | 4,490 |

Extracts were prepared from cells grown for 6 hours in the absence of L-methionine. The reaction system contained 30 μmoles potassium phosphate buffer (pH 8.5), 1.0 μmole $H_4$PteGlu, 0.1 μmole pyridoxal-5'-phosphate, 0.1 μCi DL-serine-3-$^{14}$C (1 μCi/.117 μmole), 0.1 ml cell-free extract and L-methionine as indicated, in a total volume of 0.4 ml. The assay procedure was as described in the Materials and Methods.

supplemented with methionine, which was, therefore, the sole exogenous source of methylated compounds. Under these conditions, biosynthesis of methionine would conceivably be blocked as indicated from the previous experiments. Furthermore, as methionine caused marked changes in the pteroylglutamate levels, it is possible that the pools of other related amino acids may be changed during growth under these conditions. The levels of the free amino acids in cells cultured with and without a supplement of L-methionine were, therefore, examined in further experiments. The data obtained are summarized in Tables 11 and 12.

Although the total levels of amino acids were only slightly altered in the presence of L-methionine, several individual amino acids were markedly changed by this supplement. For example, the methionine-grown cells contained relatively high levels of methionine but low levels of lysine. Changes were also observed in the levels of glutamic acid, threonine, glycine and leucine.

In further analyses, the levels of major amino acids, related to one-carbon metabolism, were examined at the different states of growth (Table 12). It is clear that methionine-grown cells contained relatively high levels of methionine throughout. In contrast, cells grown without methionine contained low but detectable levels of this amino acid. Differences in the levels of cystathionine and glycine (Table 12) were also observed. The results of these analyses indicate that amino acid pool sizes are, to some extent,

TABLE 11. *LEVELS OF AMINO ACIDS IN CELL-FREE EXTRACTS FROM DIFFERENT CULTURE MEDIA*
(after 6 hours incubation)

| N-compounds (µmoles/g.d.wt.) | Culture with methionine (2.5 µmoles/ml) Expt. 1 | Expt. 2 | Culture without methionine Expt.1 | Expt. 2 |
|---|---|---|---|---|
| Lysine | 34.0 | 52.0 | 69.4 | 94.0 |
| Histidine | 4.1 | 2.2 | 5.3 | 2.8 |
| $NH_4^+$ | 32.3 | 27.0 | 41.0 | 32.3 |
| Arginine | 10.0 | 17.0 | 21.0 | 28.4 |
| Aspartic acid | 14.0 | 20.8 | 18.1 | 13.5 |
| Threonine | 16.3 | 22.8 | 20.2 | 32.4 |
| Serine | 40.5 | 54.0 | 45.9 | 35.4 |
| Glutamic acid | 44.0 | 35.2 | 50.8 | 61.3 |
| Proline | 84.3 | 73.1 | 83.8 | 69.6 |
| Glycine | 10.6 | 14.8 | 16.0 | 20.5 |
| Alanine | 16.1 | 22.4 | 18.0 | 22.8 |
| Half cysteine | 4.0 | 4.6 | 5.0 | 7.0 |
| Valine | 8.4 | 9.8 | 7.5 | 9.9 |
| Methionine | 2.0 | 3.4 | 0.7 | 0.9 |
| Isoleucine | 1.6 | 3.6 | 2.0 | 3.6 |
| Leucine | 2.5 | 4.4 | 5.6 | 6.9 |
| Tyrosine | 1.0 | 1.7 | 2.0 | 0.5 |
| Phyenylalanine | 0.4 | 0.9 | 1.0 | 0.3 |
| Cystathionine | 9.6 | 6.5 | 11.0 | 8.2 |
| Total | 335.7 | 376.2 | 424.3 | 450.3 |

TABLE 12. *LEVELS OF SOME AMINO ACIDS IN CELL-FREE EXTRACTS FROM DIFFERENT CULTURE MEDIUM AT DIFFERENT STATES OF GROWTH*

| Compounds (µmoles/g.d.wt.) | Culture with methionine (2.5 µmole/ml) 6 hr | 12 hr | 24 hr | Culture without methionine 6 hr | 12 hr | 24 hr |
|---|---|---|---|---|---|---|
| Histidine | 3.2 | 3.1 | 4.6 | 4.1 | 3.8 | 3.9 |
| Threonine | 19.6 | 25.0 | 15.7 | 26.3 | 34.0 | 24.3 |
| Serine | 47.3 | 70.0 | 43.3 | 40.7 | 68.6 | 38.2 |
| Glycine | 12.7 | 23.0 | 13.8 | 18.3 | 29.2 | 23.1 |
| Methionine | 2.7 | 5.9 | 5.7 | 0.8 | 1.1 | 3.7 |
| Cystathionine | 8.1 | 20.7 | 17.9 | 9.6 | 13.5 | 7.5 |

altered by including L-methionine in the nutrient medium.

*Sodium Formate-$^{14}C$ Feeding Experiments*

The results of the pteroylglutamate analyses and enzyme studies using cell-free extracts suggests that the decreases in pteroylglutamate pool size, associated with methionine feeding, were not due to a greater utilization of pteroylglutamates. In the presence of methionine, it appears that there is less carbon flow through the pteroylglutamate pool mainly as a result of inhibition of 5,10-$CH_2$=$H_4$PteGlu reductase and repression of 5-$CH_3$-$H_4$PteGlu homocysteine transmethylase by this amino acid. To test the validity of this suggestion, sodium formate-$^{14}C$ was supplied to the cells and after a period of incubation, extracts were prepared for determination of $^{14}C$ present in the products of formate metabolism.

Although attempts were made to examine the incorporation of formate-$^{14}C$ into pteroylglutamate derivatives, the $^{14}C$ in these compounds was too low to measure quantitatively. However, determination of $^{14}C$ in individual free amino acids showed that formate-$^{14}C$ was readily incorporated into various amino acids. The incorporation of $^{14}C$ into these amino acids on the basis of dry weight is shown in Table 13. Serine, threonine, methionine and cystathionine were all heavily labelled. It is clear, from Table 13, that when methionine was present in the medium, $^{14}C$ incorporated into this amino acid, was only 46-60% of that found in the cells grown in

TABLE 13. *INCORPORATION OF FORMATE-$^{14}C$ INTO AMINO ACIDS IN THE PRESENCE AND ABSENCE OF L-METHIONINE IN THE CULTURE MEDIUM*

| Amino acid | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | + meth | - meth | + meth | - meth | + meth | - meth | + meth | - meth |
| Threonine | 48,500 | 32,500 | 66,000 | 37,000 | 112,100 | 54,700 | 111,100 | 135,000 |
| Serine | 224,300 | 84,000 | 222,200 | 138,400 | 290,000 | 179,600 | 359,500 | 472,200 |
| Methionine | 8,800 | 15,000 | 11,000 | 48,400 | 13,500 | 24,100 | 46,900 | 102,300 |
| Cystathionine | 8,100 | 5,400 | 14,700 | 18,900 | 18,500 | 32,800 | 174,500 | 269,200 |

Data are expressed as cpm/g.d.wt.

Cells were cultured for 3 hours with (2.5 µmole/ml) and without L-methionine supplement. Formate-$^{14}C$ (1 µc/0.023 µmole) was added directly to the cultures followed by harvesting of the cells as indicated. Individual amino acids were separated using a Beckman Model 121 Automatic Amino Acid Analyzer.

the absence of this amino acid. In contrast, greater incorporations of $^{14}C$ into serine were observed in the methionine-grown cells. Thus it appears that formate carbon was to some extent diverted from methionine synthesis when this amino acid was supplied in the medium. It is interesting to note that the amounts of $^{14}C$ incorporated into threonine and cystathionine were also different in the presence of exogenous methionine. In the methionine-grown cells, greater amounts of $^{14}C$ were incorporated into threonine but smaller amounts of $^{14}C$ were found in cystathionine. These results also indicate that the biosynthesis of these amino acids may be regulated by methionine.

In further experiments, the levels of $^{14}C$ in SAM and SAHC were examined using the techniques described in the Materials and Methods. The SAM isolated from Dowex 50X-$Na^+$ was identified by thin layer chromatography. The $R_f$ value of SAM was 0.19 in *n*-butanol:acetic acid:water (70:9:21 v/v/v) as developing solvent and 0.17 when the solvent was *n*-propanol:ammonium hydroxide:water (70:9:21 v/v/v). SAHC had $R_f$ values of 0.36 and 0.53 respectively when chromatographed in these solvent systems (Dodd, 1969). The results of these studies (Table 14) show that the level of SAM in methionine-grown cells was approximately 5 times that of cells grown without methionine. These results are in agreement with recent reports by Shapiro and Ehninger (1969) for the yeast *Candida utilis*. SAHC could not be detected in these extracts by the spectrophotometric procedure.

TABLE 14. *INCORPORATION OF FORMATE-$^{14}C$ INTO S-ADENOSYLMETHIONINE IN THE PRESENCE AND ABSENCE OF L-METHIONINE IN THE CULTURE MEDIUM*

| Feeding Period | 5 min | | 15 min | | 30 min | | 60 min* | |
|---|---|---|---|---|---|---|---|---|
| | + meth | - meth | + meth | - meth | + meth | - meth | + meth | - meth |
| SAM level (μmoles) | 5.20 | 1.0 | 5.40 | 1.01 | 5.42 | 1.02 | 5.44 | 1.02 |
| $^{14}C$ in SAM (cpm) | 137,600 | 81,700 | 314,400 | 175,200 | 699,000 | 283,000 | 848,100 | 293,700 |
| Specific activity of SAM (cpm/μmole) | 26,470 | 81,740 | 54,210 | 173,470 | 129,430 | 279,750 | 157,060 | 287,980 |
| $^{14}C$ in SAHC** (cpm) | 30,000 | 9,500 | 34,000 | 20,000 | 95,000 | 64,000 | 109,000 | 71,000 |

* Samples of S-adenosylmethionine were degraded by alkali treatment at 95°C. The resulting methionine and adenine were separated by thin layer chromatography. In methionine supplemented cells, 99% of the recovered $^{14}C$ was isolated in adenine. In the control cells, this figure was reduced to 77.5%.

** Incorporation of $^{14}C$ in SAHC was calculated by substracting the $^{14}C$ in 6N fraction of Dowex 50W($Na^+$) column from that in 6N fraction of Dowex 50W($H^+$) column.

Formate-$^{14}C$ was heavily incorporated into SAM after only 5 minutes incubation. The total $^{14}C$ in SAM was greatest when the cells were grown in the presence of methionine. However, the specific radioactivity of this compound was always higher in cells grown in the absence of methionine. $^{14}C$ was also incorporated into SAHC in these experiments, as judged by the recoveries of radioactivity from the $Na^+$ and $H^+$ columns (Shapiro and Ehninger, 1966).

In order to determine the distribution of $^{14}C$ in the methionine and adenosyl moieties of SAM, samples of this product, isolated from cells incubated with formate-$^{14}C$ for 1 hour, were degraded by alkali hydrolysis (Parks and Schlenk, 1958). Thin layer chromatography (*n*-propanol: ammonia hydroxide:water (70:9:21 v/v/v) of the hydrolysis products, revealed that the SAM isolated from methionine-grown cells contained 99% of $^{14}C$ in the adenosyl moiety. Cells cultured in the basal medium (without methionine) contained SAM-$^{14}C$ which was 23% labelled in the adenosyl moiety and 77% labelled in the methionine moiety.

# DISCUSSION

As reviewed in the Introduction, enzyme repression and product inhibition are the principal mechanisms for regulation of metabolic pathways. In *Saccharomyces cerevisiae*, methionine appears, from the present studies, to play an important regulatory role in the metabolism of one-carbon units. This amino acid appears to regulate one-carbon metabolism by inhibiting and repressing the enzymes involved in the formation and utilization of methyl groups. In the following discussion, the involvement of methionine in the control of one-carbon metabolism and the related syntheses of threonine and cystathionine are emphasized.

## *Changes in pteroylglutamate pool size as a result of enzyme repression and inhibition*

The experimental results in Figure 4a and b show that the levels of pteroylglutamates were changed during growth. These changes appear to be associated with the physiological state of the cells. Levels of pteroylglutamates increased rapidly during early logarithmic growth to reach maxima followed by decreases to levels which were most pronounced as the cells entered the post-exponential phase of growth. Such increases in pteroylglutamate levels are conceivably related to the rapid synthesis of cellular constituents occurring in the actively dividing cells. The results also indicate that levels of pteroylglutamates were finely

controlled by exogenous supplies of methionine (Figures 2, 3, 4 and 5). This is substantiated in the finding (Figures 4 and 5) that pteroylglutamate levels were rapidly increased when the cells were transferred to the basal medium without methionine. In several respects, these results are similar to those reported for animals by Noronha and Silverman (1962). In their studies, they observed that dietary administration of methionine resulted in decreases in 5-$CH_3$-$H_4$PteGlu, and suggested that methionine favored the conversion of liver 5-$CH_3$-$H_4$PteGlu to HCO-$H_4$PteGlu. Other workers in this general area have shown that methionine abolishes the excretion of formiminoglutamic acid in folic acid or vitamin $B_{12}$ deficient animals (Fox *et al.*, 1961; Silverman and Pitney, 1958). These results agree with the hypothesis that methionine promotes the conversion of 5-$CH_3$-$H_4$PteGlu to $H_4$PteGlu. Several hypotheses have, in fact, been proposed to explain this overall effect of methionine. They may be summarized as follows:

1. Methionine is converted to an acceptor capable of trapping methyl groups from the pteroyl-glutamate pool (Silverman and Pitney, 1958).
2. The higher level of S-adenosylmethionine which conceivably results from methionine feeding, increases the utilization of 5-$CH_3$-$H_4$PteGlu by stimulating the $B_{12}$ pathway of methionine formation (Kisliuk, 1964).
3. Methionine exerts a feedback inhibition on

the enzymes required for the synthesis of 5-$CH_3$-$H_4$PteGlu (Fox *et al.*, 1961).

In yeast, the reduction of the levels of pteroylglutamates by exogenously supplied methionine, cannot be explained by hypothesis 1 or 2. According to hypothesis 1, the amount of 5-$CH_3$-$H_4$PteGlu would decrease as a result of trapping by a methyl group acceptor derived from methionine. As a result, decreases in the levels of 5-$CH_3$-$H_4$PteGlu and increases in $H_4$PteGlu, and possibly also formylpteroylglutamates, should occur. However, in the present studies, all of the principal derivatives were reduced by feeding methionine (Figures 7, 8; Tables 5, 6). Furthermore, as will be discussed below, methionine was found to inhibit the biogenesis of methyl groups, a situation contrary to that postulated in hypothesis 1. Hypothesis 2 also appears unlikely as the earlier work of Pigg *et al.* (1962) has shown that yeast cells grown in methionine-rich media have less capacity to synthesize this amino acid.

The present work tends to support the third hypothesis, namely, that exogenous methionine decreases the flow of one-carbon units through the methylpteroylglutamate pool by affecting the levels and activities of pteroylglutamate enzymes. The dramatic decrease in the levels of methylated pteroylglutamates in the presence of methionine (Figure 7; Tables 5, 6) correlated with the inhibition of 5,10-$CH_2$=$H_4$PteGlu reductase by this amino acid (Figure 9), suggests that methionine exerts its effect by inhibition of the

reductase and thereby reduces the biogenesis of methyl groups within the pteroylglutamate pool. Repression of 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase by methionine in yeast (Figure 10) further indicates that stimulation of methyl group utilization for methionine biosynthesis (hypothesis 2) would not occur in this organism.

The conclusions based on pteroylglutamate analyses and enzyme studies are also substantiated by the results of the formate-$^{14}$C feeding experiments. As 10-HCO-$H_4$PteGlu synthetase was active in yeast cells (Table 7), one-carbon units from formate-$^{14}$C would conceivably enter the pteroyl-glutamate pool at this oxidation level. Incorporation of formate-$^{14}$C into methionine would demonstrate a flow of one-carbon units through the pteroylglutamate pool. It is clear from the data (Table 13), that methionine-grown cells had less capacity to convert formate-$^{14}$C into methionine.

In comparing the effects of methionine on one-carbon metabolism in yeast with other organisms, it is of interest to note that the regulatory roles of methionine in *Saccharomyces* are more complex and essentially different from those documented for *E. coli* by Taylor *et al.* (1966) and higher plants by Dodd and Cossins (1970). On the other hand, these mechanisms are, in part, similar to those reported for mammalian systems by Kutzbach and Stokstad (1967). In *E. coli*, methionine controls one-carbon metabolism by repression of 5,10-$CH_2$=$H_4$PteGlu reductase. In mammalian systems, this enzyme is not repressed but is inhibited by

SAM. In plants, regulation of methionine biosynthesis is apparently achieved by inhibition of 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase by methionine (Dodd and Cossins, 1970).

*Other aspects of one-carbon metabolism which are regulated by exogenous supplies of L-methionine*

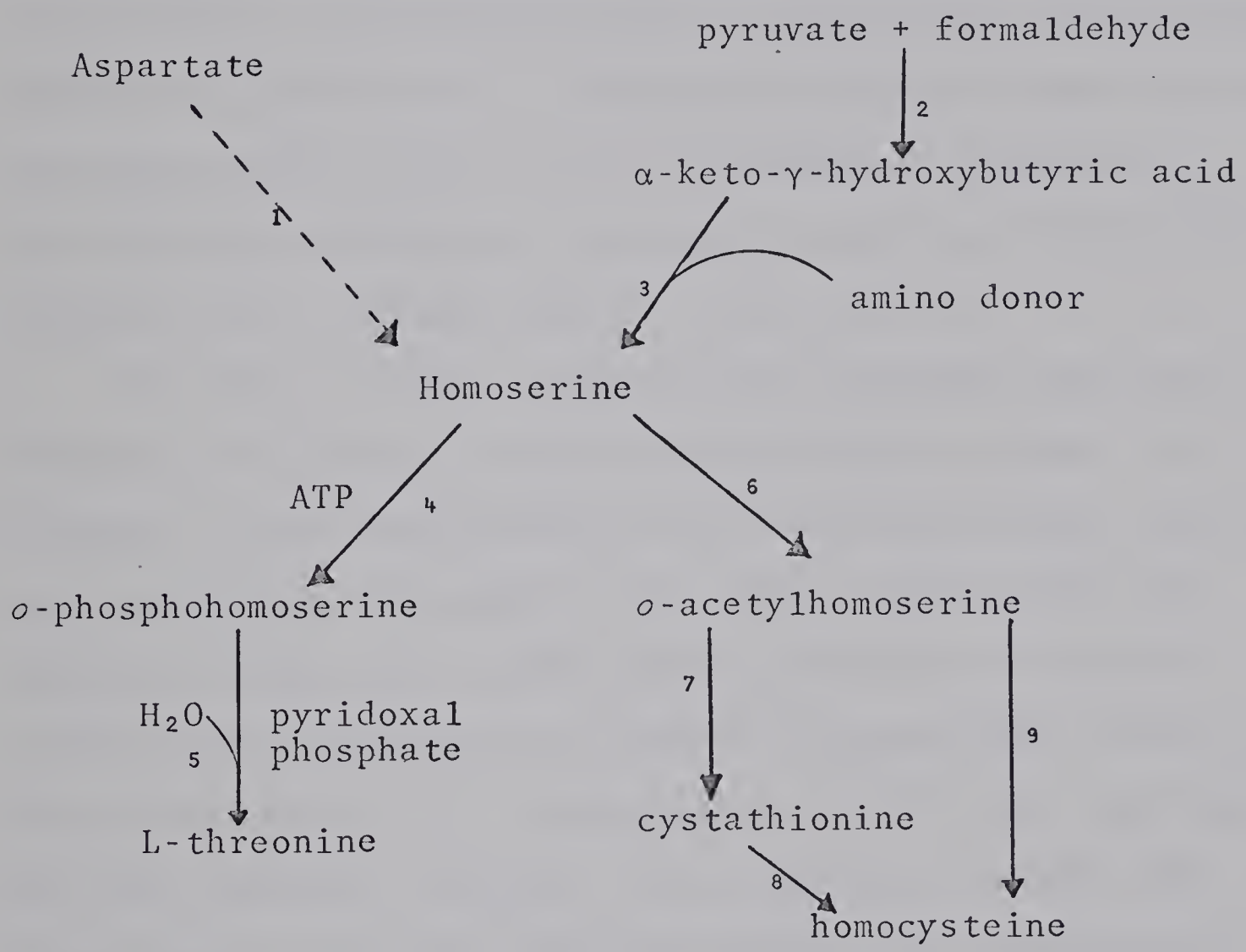
The results of the formate-$^{14}C$ feeding experiments (Table 13) further indicate that in the presence of methionine the flow of one-carbon units was diverted from methyl group biosynthesis to the synthesis of serine and adenine. It is clear from the data in Table 13, that when the basal medium was supplemented with methionine the cells contained larger amounts of $^{14}C$ in serine and the adenosyl moiety of SAM. These greater incorporations of $^{14}C$ are conceivably related to the inhibition of 5,10-$CH_2$=$H_4$PteGlu reductase by methionine. Thus diversion of one-carbon units occurred to support increased synthesis of serine and adenine, the latter being required to support the increased synthesis of SAM (Table 14).

The results of enzyme studies show that serine hydroxymethyltransferase is neither repressed nor inhibited by methionine (Tables 7 and 10). Although inhibition of this enzyme by SAM has been observed by Botsford and Parks (1969), this may not be of physiological importance as the results of the formate-$^{14}C$ feeding experiments (Table 13) show that this reaction proceeded more readily in cells which were

accumulating SAM (Table 14). Although 10-HCO-$H_4$PteGlu synthetase activity was inhibited to some extent at high concentrations of methionine (Table 9), this effect is also of doubtful physiological significance as a method for regulating the biosynthesis of pteroylglutamate derivatives mainly because 10-HCO-$H_4$PteGlu is directly involved in purine and protein synthesis. Furthermore, as the majority of the $^{14}C$ in SAM of methionine-grown cells was associated with the adenosyl moiety, it appears unlikely that inhibition or repression of this enzyme by methionine occurs *in vivo*.

The results of the formate-$^{14}C$ feeding experiments also indicate that the pathway leading to the biosynthesis of threonine and cystathionine may be controlled by methionine. This pathway is related to the biosynthesis of homocysteine, the final methyl acceptor in methionine biosynthesis. Threonine and cystathionine have a common origin from homoserine which may in turn be derived from one of two pathways (Scheme 3). Black and Wright (1955) demonstrated the conversion of aspartate to homoserine in cell-free extracts of baker's yeast (Scheme 3, reaction 1). In preparations of beef liver, formation of α-keto-γ-hydroxybutyric acid from pyruvate and formaldehyde (Scheme 3, reaction 2; Hift and Mahler, 1952) and transamination of this α-keto acid leading to the formation of homoserine (Scheme 3, reaction 3) have been observed (Meister, 1956). In *Saccharomyces*, homoserine is further converted to threonine via the intermediary formation of *o*-phosphohomoserine, steps which are catalyzed

SCHEME 3. *PATHWAYS INVOLVED IN THE BIOSYNTHESIS OF THREONINE, CYSTATHIONINE AND HOMOCYSTEINE*



by homoserine kinase (Scheme 3, reaction 4; Watanabe *et al.*, 1957) and threonine synthetase (Scheme 3, reaction 5; Watanabe and Shimura, 1960), respectively. Similar reactions are operative in *E. coli* (Patte *et al.*, 1963) and *Neurospora crassa* (Flavin and Slaughter, 1960). Homoserine can also be converted to *o*-acetylhomoserine via homoserine transacetylase (Scheme 3, reaction 6). *o*-Acetylhomoserine is then converted to homocysteine either via the intermediary formation of cystathionine (Scheme 3, reactions 7 and 8) or directly by reaction with sulfide (Scheme 3, reaction 9).

The data in Table 13 shows that threonine and cystathionine were heavily labelled with formate carbon. If formate-$^{14}C$ was incorporated into threonine through aspartate as described by Watanabe *et al.* (1957) and Watanabe and Shimura (1960), this latter amino acid should have been labelled particularly as it occurs in appreciable levels in the cells (Table 11). Incorporation of $^{14}C$ into aspartate was not, however, observed. It is conceivable that the labelling of threonine might be derived via pyruvate and formaldehyde, reactions which occur in liver preparations (Hift and Machler, 1952; Meister, 1956). Formate-$^{14}C$ could be converted to formaldehyde-$^{14}C$ via the intermediary formation of 5,10-$CH_2$=$H_4$PteGlu. The lack of labelling in homoserine is surprising, but may be associated with the extremely small pool size of this amino acid.

It is interesting to note that the amounts of $^{14}C$ in threonine and cystathionine were changed when methionine was

supplied. In the methionine-grown cells greater amounts of $^{14}C$ were accumulated in threonine but relatively small amounts of radioactivity were found in cystathionine (Table 13). It is not surprising that in the presence of methionine, threonine was heavily labelled from formate-$^{14}C$ because induction of homoserine kinase by exogenous methionine has been observed in yeast (de Robichon-Szulmajster, 1971). Decreases in the incorporation of formate-$^{14}C$ into cystathionine associated with the presence of exogenous methionine suggests that the formation of this intermediate is controlled by methionine. This may be achieved by regulation of *o*-acetyl homoserine synthesis (de Robichon-Szulmajster, 1971) which in turn would control the production of homocysteine. However, the involvement of cystathionine as an intermediate in methionine synthesis in *Saccharomyces* has not been fully elucidated and is questionable as only low levels of cystathionine synthase have been detected in this organism (Kerr and Flavin, 1970). Also, there is evidence indicating that the sulfhydrylase reaction which by-passes the formation of cystathionine is the major route of methionine biosynthesis in *Saccharomyces* (Cherest *et al.*, 1969). Clearly, the significance of this effect of methionine on cystathionine synthesis is still to be determined.

It is clear from the above discussion that inhibition of 5,10-$CH_2$=$H_4$PteGlu reductase and the partial repression of 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase by methionine cannot account for the overall decreases in pteroylglutamates

(Figures 2 and 3) which occurred when the cells were cultured in the presence of exogenous methionine. As metabolism of one-carbon units involves a highly branched pathway, feed-back inhibition or repression of pteroylglutamate formation by one end-product could affect the levels of other metabolically related derivatives. Therefore, it is conceivable that the net biosynthesis of pteroylglutamates may be indirectly regulated by such end-products or more directly by a product of methionine metabolism. Such regulation would allow the cell to utilize the low levels of pteroylglutamates in reactions which are of immediate importance to the cell. Thus, in the presence of methionine, the biogenesis of methyl groups would be specifically regulated. Regulation of 5,10-$CH_2$=$H_4$PteGlu reductase by methionine would clearly be of prime importance in this respect because the synthesis of 5-$CH_3$-$H_4$PteGlu may not be reversible under physiological conditions. In addition, the repression of 5-$CH_3$-$H_4$PteGlu: homocysteine transmethylase by methionine would provide a further mechanism for regulating flow of these one-carbon units from the pteroylglutamate pool. Under these conditions, 5,10-$CH_2$=$H_4$PteGlu would be available for alternative pathways which may, in turn, be regulated by their end-products. Indeed, other end-products of the pteroylglutamate pathway, such as purines and serine, have been shown to be important regulators of one-carbon metabolism in certain bacteria (Burchall and Hitchings, 1965; Albrecht *et al.*, 1966). Further studies with *Saccharomyces* are required to determine

whether these regulatory mechanisms are also operative in this species.

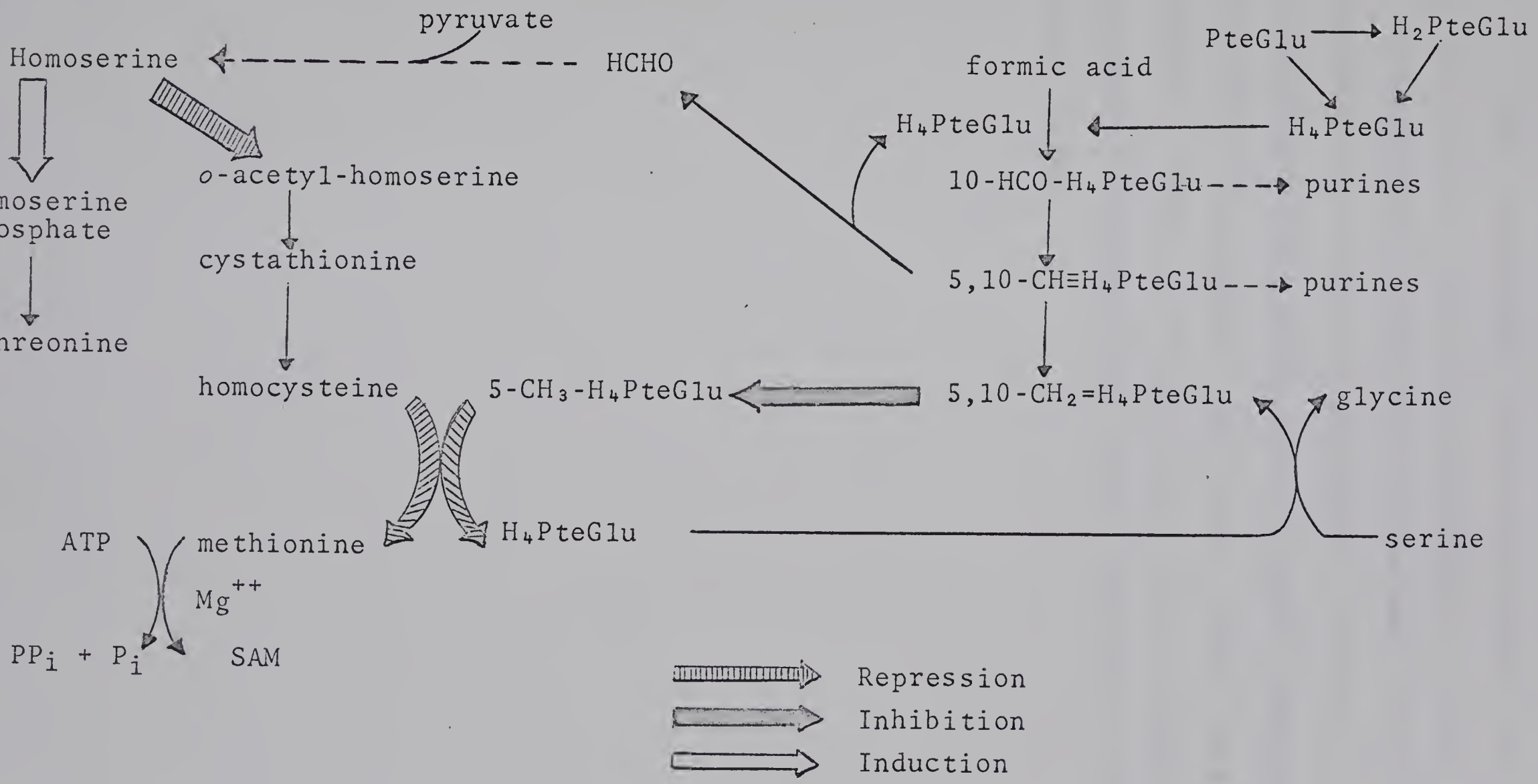
*Concluding remarks*

The results of the present work clearly indicate that methionine plays an important role in regulating the biogenesis and utilization of methyl groups from the pteroylglutamate pool. This amino acid decreases the flow of carbon through methyltetrahydropteroylglutamate by inhibition of 5,10-$CH_2$=$H_4$PteGlu reductase and repression of 5-$CH_3$-$H_4$PteGlu: homocysteine transmethylase. The availability of homocysteine, a methyl acceptor for the transmethylase reaction involved in methionine biosynthesis, may also be regulated by this amino acid. These basic conclusions are summarized in Scheme 4.

At present, knowledge of one-carbon metabolism is still incomplete, this is particularly true of information regarding the basic mechanisms controlling production and utilization of pteroylglutamates. The present results have focussed attention on mechanisms for regulation of methyl pteroylglutamates. However, questions arising from the present work, such as the actual mechanisms for regulation of 5,10-$CH_2$=$H_4$PteGlu reductase and the genetic basis for regulation of 5-$CH_3$-$H_4$PteGlu:homocysteine transmethylase by methionine, remain to be fully elucidated.

Regulation of one-carbon metabolism may also be provided by the intracellular localization of related

SCHEME 4. *POSSIBLE REGULATORY ROLES OF METHIONINE ON ONE-CARBON METABOLISM IN SACCHAROMYCES CEREVISIAE*



enzymes. In this connection, it is conceivable that the rate and direction of one-carbon metabolism in living cells may be, to some extent, controlled by movement of substrates and products between these compartments. As there is now evidence that one-carbon metabolism is highly compartmented in higher plants and animals (Shah *et al.*, 1970; Wang *et al.*, 1967), there is reason to believe that various aspects of pteroylglutamate metabolism are finely regulated at the sub-cellular level.

# BIBLIOGRAPHY

ALBRECHT, A.M. and HUTCHISON, D.J. 1964. Repression by adenine of the formyltetrahydrofolate synthetase in an antifolic-resistant mutant of *Streptococcus faecalis*. *J. Bacteriol.* 87: 795-798.

ALBRECHT, A.M., PEARCE, F.K. and HUTCHISON, D.J. 1966. Metabolic control of 5,10-methylenetetrahydrofolate dehydrogenase formation in *Streptococcus faecalis*. *Bact. Proc.* p. 97.

ALLFREY, V.G. and KING, C.G. 1950. An investigation of the folic acid-protein complex in yeast. *J. Biol. Chem.* 182: 367-384.

ANGIER, R.B., BOOTHE, J.H., HUTCHINGS, B.L., MOWAT, J.H., SEMB, J., STOKSTAD, E.L.R., SUBBA ROW, Y., WALLER, C.W., COSULICH, D.G., FAHRENBACH, M.J., HULTGUIST, M.E., KUH, E., NORTHEY, E.H., SEEGER, D.R., SICKELS, J.P. and SMITH, J.M. Jr. 1946. The structure and synthesis of the liver *L. casei* factor. *Science* 103: 667-669.

BALISH, E. and SHAPIRO, S.K. 1967. Methionine biosynthesis in *Escherichia coli*: Induction and repression of methylmethionine (or adenosylmethionine):homocysteine methyltransferase. *Arch. Biochem. Biophys.* 119: 62-68.

BAKERMAN, H.A. 1961. A method for measuring the microbiological activity of tetrahydrofolic acid and

other labile reduced folic acid derivatives. *Anal. Biochem.* 2: 558-567.

BERTINO, J.R., PERKINS, J.P. and JOHNS, D.G. 1965. Purification and properties of dihydrofolate reductase from *Ehrlich ascites carcinoma* cells. *Biochem.* 4: 839-846.

BERTINO, J.R., SILBER, R., FREEMAN, M., ALENTY, A., ALBRECHT, M., GABRIO, B.W. and HUENNEKENS, F.M. 1963. Studies on normal and leukemic leukocytes. IV. Tetrahydrofolate-dependent enzyme systems and dihydrofolic reductase. *J. Clin. Invest.* 42: 1899-1907.

BERTINO, J.R., SIMMONS, B. and DONOHUE, D.M. 1964. Levels of dihydrofolate reductase and the formate-activating enzyme activities in guinea pig tissues. *Biochem. Pharmacol.* 13: 225-233.

BIRD, O.D., BINKLEY, S.B., BLOOM, E.S., EMMETT, A.D. and PFIFFNER, J.J. 1945. On the enzymic formation of Vitamin $B_C$ from its conjugate. *J. Biol. Chem.* 157: 413-414.

BIRD, O.D., McGLOHON, V.M. and VAITKUS, J. 1965. Naturally occurring folates in the blood and liver of the rat. *Anal. Biochem.* 12: 18-35.

BIRD, O.D., ROBBINS, M., VANDENBELT, J.M. and PFIFFNER, J. J. 1946. Observations of vitamin $B_C$ conjugase from hog kidney. *J. Biol. Chem.* 163: 649-659.

BLAKLEY, R.L. 1954. The interconversion of serine and

glycine. Role of pteroylglutamic acid and other cofactors. *Biochem. J.* 58: 448-462.

BLAKLEY, R.L. 1969. In: *The Biochemistry of Folic Acid and Related Pteridines.* (ed.) Blakley, R.L., American Elsevier Pub. Co. Inc., New York, N.Y. p. 267.

BLACK, S. and WRIGHT, N.G. 1955. Homoserine dehydrogenase. *J. Biol. Chem.* 213: 51-60.

BOTSFORD, J.L. Jr. and PARKS, L.W. 1969. Serine trans-hydroxymethylase in methionine biosynthesis in *Saccharomyces cerevisiae. J. Bact.* 97: 1176-1183.

BRAGANCA, B.M. and KENKARE, U.W. 1964. Folic acid reductases in relation to normal and malignant growth. *Acta Unio. Int. Cancer* 20: 980-982.

BREMER, J. and GREENBERG, D.M. 1961. Methyl transfering enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholine). *Biochem. Biophys. Acta* 46: 205-216.

BURCHALL, J.J. and HITCHINGS, G.H. 1965. Inhibitor binding analysis of dihydrofolate reductase from various species. *Mol. Pharmacol.* 1: 126-136.

BURCHALL, J.J. and HITCHINGS, G.H. 1967. The role of metabolites and antimetabolites in the control of folate coenzyme synthesis. *Adv. Enzyme Reg.* 6: 323-333.

BURTON, E., SELHUB, J. and SAKAMI, W. 1969. The substrate specificity of 5-methyltetrapteroyltriglutamate-

homocysteine methyltransferase. *Biochem. J.* 111: 793-795.

CANTONI, G.L. and DURELL, J. 1957. Activation of methionine for transmethylation. *J. Biol. Chem.* 225: 1033-1048.

CATHOU, R.E. and BUCHANAN, J.M. 1963. Enzymatic synthesis of the methyl group of methionine. V. Studies with 5,10-methylenetetrahydrofolate reductase from *E. coli*. *J. Biol. Chem.* 238: 1746-1751.

CHAN, Pak-Yu. 1971. Regulation of the Krebs-Henseleit cycle in *Saccharomyces cerevisiae*. M.Sc. thesis, University of Alberta, Edmonton, Alberta, Canada.

CHEREST, H., EICHLER, F. and de ROBICHON-SZULMAJSTER, H. 1969. Genetic and regulatory aspects of methionine biosynthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 97: 328-336.

COMBEPINE, G., COSSINS, E.A. and LOR, K.L. 1971. Regulation of pteroylglutamate pool size by methionine. *FEBS Letters* 14: 49-53.

COSSINS, E.A. and SHAH, S.P.J. 1971. Pteroylglutamates of higher plant tissues: Localization of tetrahydropteroylglutamate derivatives in chloroplasts. *Phytochemistry* (in press).

COSSINS, E.A. and SINHA, S.K. 1966. The interconversion of glycine and serine by plant tissue extracts. *Biochem. J.* 101: 542-549.

COSSINS, E.A., WONG, K.F. and ROOS, A.J. 1970. Plant $N^5$, $N^{10}$-methylenetetrahydrofolate dehydrogenase. Partial purification and some general properties of the enzyme from germinating pea seedlings. *Phytochem.* 9: 1463-1471.

CROPPER, W.J. and SCOTT, M.L. 1966. Nature of the blood folates in young chicks and poults fed pteroylmonoglutamic acid or pteroylheptaglutamates (31259). *Proc. Soc. Exptl. Biol. Med.* 122: 817-820.

DALAL, F.R. and GOTS, J.S. 1966. Inhibition of 5,10-methylenetetrahydrofolate dehydrogenase by purine nucleotides. *Biochem. Biophys. Res. Commun.* 22: 340-345.

DALAL, F.R. and GOTS, J.S. 1967. Purification of 5,10-methylenetetrahydrofolate dehydrogenase from *Salmonella typhimurium* and its inhibition by purine nucleotides. *J. Biol. Chem.* 242: 3636-3640.

DELAVIER-KLUTCHKO, C. and FLAVIN, M. 1965. Enzymatic synthesis and cleavage of cystathionine in fungi and bacteria. *J. Biol. Chem.* 240: 2537-2549.

deROBICHON-SZULMAJSTER, H. 1971. Diversity of regulation mechanisms involved in threonine and methionine biosynthesis in *Saccharomyces cerevisiae*. *Biochimie* 53: 131-134.

DICKERMAN, H. and WEISSBACH, H. 1964. Altered folate metabolism in a vitamin $B_{12}$-methionine auxotroph. *Biochm. Biophys. Res. Commun.* 16: 593-599.

DOCTOR, V.M. and COUCH, J.R. 1953. Occurrence and properties of a conjugated form of *Leuconostoc citrovorum* factor. *J. Biol. Chem.* 200: 223-231.

DODD, W.A. and COSSINS, E.A. 1970. Homocysteine-dependent transmethylases catalyzing the synthesis of methionine in germinating pea seeds. *Biochim. Biophys. Acta* 201: 461-470.

DONALDSON, K.O. and KERESZTESY, J.C. 1962. Naturally occurring forms of folic acid. II. Enzymatic conversion of methylenetetrahydrofolic acid to prefolic A-methyltetrahydrofolate. *J. Biol. Chem.* 237: 1298-1304.

DONALDSON, K.O., SCOTT, V.F. and SCOTT, W. 1965. Purification and properties of 5,10-methylenetetrahydrofolate dehydrogenase from *E. coli*. *J. Biol. Chem.* 240: 4444-4448.

EIGEN, E. and SHOCKMAN, G.D. 1963. In: *Analytical Microbiology*. (ed.) Kavanagh, F., Academic Press, Inc., New York and London. p. 431.

ELFORD, H.L., KATZEN, H.M., ROSENTHAL, S., SMITH, L.C. and BUCHANAN, J.M. 1965. In: *Transmethylation and Methionine Biosynthesis*. (ed.) Shapiro, S.K. and Schlenk. The University of Chicago Press. p. 157.

FLAVIN, M. and SLAUGHTER, C. 1960. Purification and properties of threonine synthetase of *Neurospora*. *J. Biol. Chem.* 235: 1103-1108.

FOSTER, M.A., TEJERINA, G., GUEST, J.R. and WOODS, D.D.

1964. Two enzymic mechanisms for the methylation of homocysteine by extracts of *Escherichia coli*. *Biochem. J.* 92: 476-488.

FOX, M.R.S., LUDWING, W.J. and BAROODY, M.C. 1961. Effects of vitamin $B_{12}$ and methionine on excretion of formiminoglutamic acid by the chick. *Proc. Soc. Exptl. Biol. Med.* 107: 723-727.

GILBERT, W. and MÜLLER-HILL, B. 1966. Isolation of the lac repressor. *Proc. Nat. Acad. Sci.* 56: 1891-1898.

GILBERT, W. and MÜLLER-HILL, B. 1967. The lac operator is DNA. *Proc. Nat. Acad. Sci.* 58: 2415-2421.

GOLD, M. and HURWITZ, J. 1963. Enzymatic methylation of nucleic acids. *Federation Proc.* 22: 230.

GREENBERG, D.M., TAM, B.D., JENNY, E. and PAYES, B. 1966. Highly purified dihydrofolate reductase of calf thymus. *Biochim. Biophys. Acta* 122: 423-435.

GUEST, J.R., FOSTER, M.A. and WOODS, D.D. 1964. Methyl derivatives of folic acid as intermediates in the methylation of homocysteine by *E. coli*. *Biochem. J.* 92: 488-496.

GUEST, J.R. and WOODS, D.D. 1965. The interaction of folic acid derivatives in the methylation of homocysteine. *Biochem. J.* 97: 500-512.

HAKALA, M.T. and WELCH, A.D. 1957. A polyglutamate form of *Citrovorum* factor synthesized by *Bacillus subtilis*. *J. Bacteriol.* 73: 35-41.

HATCH, F.T., LARRABEE, A.R., CATHOU, R.E. and BUCHANAN, J.M.

1961. Enzymatic synthesis of the methyl group of methionine. I. Identification of the enzymes and cofactors involved in the system isolated from *E. coli*. *J. Biol. Chem.* 236: 1095-1101.

HERBERT, V. and ZALVSKY, R. 1962. Interrelations of vitamin $B_{12}$ and folic acid metabolism: Folic acid clearance studies. *J. Clin. Invest.* 41: 1263-1276.

HIATT, A.J. 1965. Formic acid activation in plants. I. Purification properties and distribution of formyl-tetrahydrofolate synthetase. *Pl. Physiol.* 40: 184-189.

HIFT, H. and MAHLER, H.R. 1952. The enzymatic condensation of pyruvate and formaldehyde. *J. Biol. Chem.* 198: 901-914.

HILLCOAT, B.L. and BLAKLEY, R.L. 1966. Dihydrofolate reductase of *Streptococcus faecalis*. I. Purification and some properties of reductase from the wild strain and from strain A. *J. Biol. Chem.* 241: 2995-3001.

HOGAN, A.G. and PARROTT, E.M. 1940. Anemia in chicks caused by a vitamin deficiency. *J. Biol. Chem.* 132: 507-517.

IWAI, K., NAKAGAWA, S. and OKINAKA, O. 1959. Studies on folic acid group in plant tissues. XI. On the members and the forms of folinic acid group in green leaves. *Mem. Res. Inst. Food Sci.*, Kyoto University, No. 19. pp. 17-37.

IWAI, K., OKINAKA, O., IKEDA, M. and SUZUKI, N. 1969. The biosynthesis of folic acid compounds in plants. *IVth International Congress on Pteridines.*

JACOB, F. and MONOD, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3: 318-356.

JAENICKE, L. 1956. Conversion of β-carbon of serine to $N^{10}$-formyltetrahydrofolic acid. *Federation Proc.* 15: p. 281.

JONES, E.W. and MAGASANIK, B. 1967. Genetic block in the interconversions of folic acid coenzymes in *Saccharomyces cerevisiae*. *Bact. Proc.* 127:

JUKES, T.H. and STOKSTAD, E.L.R. 1948. Pteroylglutamic acid and related compounds. *Physiol. Rev.* 28: p.51.

JUKES, T.H. and WILLIAMS, W.L. 1954. In: *The Vitamins*. $B_{12}$ content of food. Vol. 1. (ed.) Sebrell, W.H. and Harris, R.S., Academic Press, Inc., New York. p. 477.

KATZEN, H.M. and BUCHANAN, J.M. 1965. Enzymatic synthesis of the methyl group of methionine. VIII. Repression-derepression, purification and properties of 5,10-methylenetetrahydrofolate reductase from *Escherichia coli*. *J. Biol. Chem.* 240: 825-835.

KAUFMAN, B.T. and GARDINER, R.C. 1966. Studies of dihydrofolate reductase. I. Purification and properties of dihydrofolate reductase from chicken liver. *J. Biol. Chem.* 241: 1319-1328.

KERR, D.S. and FLAVIN, M. 1970. The regulation of methionine

synthesis and the nature of cystathionine γ-synthase in *Neurospora*. *J. Biol. Chem.* 245: 1842-1855.

KISLIUK, R.L. 1963. The source of hydrogen for methionine methyl formation. *J. Biol. Chem.* 238: 397-400.

KISLIUK, R.L. 1964. The role of adenosylmethionine and 5-methyltetrahydrofolate in the regulation of the metabolism of single carbon units. *Medicine* 43: 711-713.

KUTZBACH, C. and STOKSTAD, E.L.R. 1967a. Purification and some properties of the allosteric methyltetrahydrofolate:NADP oxidoreductase from rat liver. *Federation Proc.* 26: p. 559.

KUTZBACH, C. and STOKSTAD, E.L.R. 1967b. Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by S-adenosylmethionine. *Biochim. Biophys. Acta* 139: 217-220.

LASKOWSKI, M., MIMS, V. and DAY, P.L. 1945. Studies on the enzyme which produces the *Streptococcus lactis* R stimulating factor from inactive precursor substance in yeast. *J. Biol. Chem.* 157: 731-739.

LAZOWSKA, J. and LUZZATI, M. 1970a. Biochemical deficiency associated with *ad*3 mutations in *Saccharomyces cerevisiae*. I. Levels of three enzymes of tetrahydrofolate metabolism. *Biochim. Biophys. Res. Commun.* 39: 34-39.

LAZOWSKA, J. and LUZZATI, M. 1970b. Biochemical deficiency associated with *ad*3 mutations in *Saccharomyces*

*cerevisiae*. II. Separation of two forms of methylenetetrahydrofolate dehydrogenase. *Biochem. Biophys. Res. Commun.* 39: 40-45.

LEDERER, E. 1965. C-methylation in biological systems. *Israel J. Med. Sci.* 1: 1129-1147.

LOWRY, O.H., ROSEBROUCH, N.J., FARR, A.L. and RANDALL, R.J. 1951. Protein measurement with the folin reagent. *J. Biol. Chem.* 193: 265-275.

MacKENZIE, C.G. 1950. Formation of formaldehyde and formate in the biooxidation of the methyl group. *J. Biol. Chem.* 186: 351-368.

MacKENZIE, C.G. and ABELES, R.N. 1956. Production of active formaldehyde in the mitochondrial oxidation of sarcosine-CD3. *J. Biol. Chem.* 222: 145-150.

MANDEL, L.R. and BOREK, E. 1961. Variability in the structure of ribonucleic acid. *Biochem. Biophys. Res. Commun.* 4: 14-18.

MANGUM, J.H. and SCRIMGEOUR, K.C. 1962. Cofactor requirements and intermediates in methionine biosynthesis. *Federation Proc.* 21: 242.

MEISTER, A. 1956. Nonoxidative and nonproteolytic enzymes (amino acid transaminases and racemases). *Ann. Rev. Biochem.* 25: 29-56.

MEISTER, A. 1965. In: *Biochemistry of the Amino Acids.* Vol. II. Academic Press, New York. p. 771.

MELL, G.P., SCHROEDER, G.H. and HUENNEKENS, F.M. 1966. Molecular properties of dihydrofolate reductase.

*Federation Proc.* 25: 277.

MIMS, V. and LASKOWSKI, M. 1945. Studies on Vitamin $B_c$ conjugase from chicken pancreas. *J. Biol. Chem.* 160: 493-503.

MIMS, V., TOTTER, J.R. and DAY, P.L. 1944. A method for the determination of substances enzymatically convertible to the factor stimulating *Streptococcus lactis* R. *J. Biol. Chem.* 155: 401-405.

MITCHELL, H.K., SNELL, E.E. and WILLIAMS, R.J. 1941. The concentration of "Folic Acid". *J. Am. Chem. Soc.* 63: p. 2284.

MONOD, J. and JACOB, F. 1961. Teleonomic mechanisms in cellular metabolism, growth, and differentiation. *Cold Spring Harbor Sym. Quant. Biol.* 26: 389.

MORNINGSTAR, J.F. Jr. and KISLIUK, R.L. 1965. Interrelations between two pathways of methionine biosynthesis in *Aerobacter aerogenes*. *J. Gen. Microbiol.* 39: 43-51.

MUDD, S.H. and CANTONI, G.L. 1958. Activation of methionine for transmethylation. *J. Biol. Chem.* 231: 481-492.

MUDD, S.H. and CANTONI, G.L. 1964. Biological transmethylation, methyl-group biogenesis and other one carbon metabolic reactions dependent upon tetrahydrofolic acid. In: *Comprehensive Biochemistry*. Vol. 15. (ed.) Forkin, M. and Stolz, E.H., Elsevier Publishing Co., Amsterdam. pp. 1-42.

NORONHA, J.M. and ABOOBAKER, V.S. 1963. Studies on the

folate compounds of human blood. *Arch. Biochem. Biophys.* 101: 445-447.

NORONHA, J.M. and SILVERMAN, M. 1962. Distribution of folic acid derivatives in natural material. I. Chicken liver folates. *J. Biol. Chem.* 237: 3299-3302.

NORONHA, J.M. and SILVERMAN, M. 1962. In: *Second European Symposium on Vitamin $B_{12}$ and Intrinsic Factor on Folic Acid Vitamin $B_{12}$, Methionine and Formimino-glutamate Metabolism.* Enke p. 728.

NURMIKKO, V., TERHO, S.T. and KANTONEN, U.M. 1964. Formation of folate enzymes during the growth cycle of bacteria. II. Activity of serine hydroxymethyltransferase during the lag, acceleration and exponential phases of growth of *E. coli*, *Streptococcus faecalis*, *Lactobacillus arabinosus* and *Streptococcus thermophilus*. *Acta Chem. Scand.* 18: 627-637.

OHARA, O. and SILBER, R. 1969. Studies on the regulation of one-carbon metabolism. The effects of folate concentration in the growth medium on the activity of three folate-dependent enzymes in *Lactobacillus casei*. *J. Biol. Chem.* 244: 1988-1993.

PARKS, L.W. and SCHLENK, F. 1958. The stability and hydrolysis of S-adenosylmethionine; isolation of S-ribosylmethionine. *J. Biol. Chem.* 230: 295-305.

PATTE, J.C., LeBRAS, G., LOVINY, T. and COHEN, G.N. 1963. End-product inhibition and repression of the homoserine dehydrogenase of *E. Coli*. *Biochim. Biophys.*

*Acta* 67: 16-30.

PIGG, C.J., SPENCE, K.D. and PARKS, L.W. 1962. Methionine biosynthesis in yeast. *Arch. Biochem. Biophys.* 97: 471-496.

RABINOWITZ, J.C. and PRICER, W.E. Jr. 1962. Formyltetrahydrofolate synthetase. I. Isolation and crystallization of the enzyme. *J. Biol. Chem.* 237: 2898-2902.

RAMASASTRI, B.V. and BLAKLEY, R.L. 1964. 5,10-methylene-tetrahydrofolic dehydrogenase from baker's yeast. *J. Biol. Chem.* 239: 106-111.

RIGGS, A.D. and BOURGEOIS, S. 1968. On the assay, isolation and characterization of the lac repressor. *J. Mol. Biol.* 34: 361-364.

ROBERTS, D. and HALL, T.C. 1963. Folic reductase content of human and animal tissues. *Proc. Am. Assoc. Cancer Res.* 4: 57.

ROHRINGER, R., KIM, W.K. and SAMBORSKI, D.J. 1969. Folate derivatives in healthy and rust-infected primary leaves of wheat. *Can. J. Biochem.* 47: 1161-1169.

ROMAN, H.L. 1956. A system selective for mutations affecting the synthesis of adenine in yeast. *C.R. Trav. Lab. Carlsberg* 26: 299-314.

ROOS, A.J. 1971. The synthesis of pteroylglutamates in germinating pea cotyledons. Ph.D. thesis, University of Alberta, Edmonton, Alberta, Canada.

ROOS, A.J. and COSSINS, E.A. 1971. Pteroylglutamate derivatives of *Pisum sativum* L. Synthesis of cotyledonary tetrahydropteroylglutamates during germination.

*Biochem. J.* (in press).

ROOS, A.J., SPRONK, A.M. and COSSINS, E.A. 1968. 5-Methyl-tetrahydrofolic acid and other folate derivatives in germinating pea seedlings. *Can. J. Biochem.* 46: 1533-1536.

ROWBURY, R.J. and WOODS, D.D. 1961. Repression of methionine synthesis in *Escherichia coli*. *J. Gen. Microbiol.* 24: 129-144.

SAKAMI, W. 1950. Formation of formate and labile methyl groups from acetone in the intact rat. *J. Biol. Chem.* 187: 369-378.

SAKAMI, W. and UKSTINS, I. 1961. Enzymic methylation of homocysteine by a synthetic tetrahydrofolate derivative. *J. Biol. Chem.* 236: PC50-51.

SCHERTEL, M.E., BOEHNE, J.W. and LIBBY, D.A. 1965. Folic acid derivatives in yeast. *J. Biol. Chem.* 240: 3154-3158.

SCHLENK, F. 1965. Biochemical and cytological studies with sulfonium compounds. In: *Transmethylation and Methionine Biosynthesis*. (ed.) Shapiro and Schlenk. University of Chicago Press, Chicago. p. 48.

SCHLENK, F. and DePALMA, R.E. 1957. The preparation of S-adenosylmethionine. *Biochim. Biophys. Acta* 140: 1051-1057.

SHAH, S.P.J., ROOS, A.J. and COSSINS, E.A. 1970. Synthesis and intracellular localization of tetrahydrofolate derivatives in higher plant tissues. In: *Chemistry*

*and Biology of Pteridines*. (ed.) Iwai, K., Akino, M., Goto, M., and Iwanami, Y., International Academic Printing Co., Tokyo. pp. 305-314.

SHAPIRO, S.K., ALMENAS, A. and THOMSON, J.F. 1965. Biosynthesis of methionine in *Saccharomyces cerevisiae*. Kinetics and mechanism of reaction of S-adenosylmethionine-homocysteine methyltransferase. *J. Biol. Chem*. 240: 2512-2518.

SHAPIRO, S.K. and EHNINGER, D.J. 1966. Methods for the analysis and preparation of adenosylmethionine and adenosylhomocysteine. *Analyt. Biochem*. 15: 323-333.

SHAPIRO, S.K. and EHNINGER, D.J. 1969. Biosynthesis of adenosylmethionine from adenosylhomocysteine in *Candida utilis*. *Biochim. Biophys. Acta* 177: 67-77.

SIEKEVITZ, P. and GREENBERG, D.M. 1950. The biological formation of formate from methyl compounds in liver slices. *J. Biol. Chem*. 186: 275-286.

SILVERMAN, M., LAW, L.W. and KAUFMAN, B. 1961. The distribution of folic acid activities in lines of leukemic cells of the mouse. *J. Biol. Chem*. 236: 2530-2533.

SILVERMAN, M. and PITNEY, A.J. 1958. Dietary methionine and the excretion of formiminoglutamic acid by the rat. *J. Biol. Chem*. 233: 1179-1182.

SIMPSON, R.E. and SCHWEIGERT, B.S. 1949. Folic acid metabolism studies. I. Occurrence of blood

conjugases. *Arch. Biochem. Biophys.* 20: p. 32.

SOTOBAYASHI, H., ROSEN, F. and NICHOL, C.A. 1966. Tetrahydrofolate cofactors in tissues sensitive and refractory to amethopterin. *Biochem.* 5: 3878-3883.

TABOR, H. and TABOR, C.W. 1960. Further purification of enzymes concerned with the biosynthesis of spermidine in *E. coli*. *Federation Proc.* 19: 6.

TAYLOR, R.T., DICKERMAN, H. and WEISSBACH, H. 1966. Control of one-carbon metabolism in a methionine-$B_{12}$ auxotroph of *Escherichia coli*. *Arch. Biochem. Biophys.* 117: 405-412.

TAYLOR, R.T. and WEISSBACH, H. 1965. Radioactive assay for serine transhydroxymethylase. *Analyt. Biochem.* 13: 80-84.

THOMPSON, J.F. 1967. Sulfur metabolism in plants. *Ann. Rev. Plant Physiol.* 18: 59-84.

VOLCANI, B.E. and MARGALITH, P. 1957. A new species (*Flavobacterium polyglutamicum*) which hydrolyzes the γ-L-glutamyl bond in polypeptides. *J. Bacteriol.* 74: 646-655.

WANG, F.K., KOCH, J. and STOKSTAD, E.L.R. 1967. Folate coenzyme pattern, folate linked enzymes and methionine biosynthesis in rat liver mitochondria. *Biochem. Z.* 346: 458-466.

WATANABE, Y., KONISHI, S. and SHIMURA, K. 1957. Biosynthesis of threonine from homoserine. VI. Homoserine kinase. *J. Biochem.* (Tokyo) 44: 299-307.

WATANABE, Y. and SHIMURA, K. 1960. Mechanism of L-threonine synthesis. *J. Biochem.* (Tokyo) 47: 266-268.

WHITELEY, H.R. 1960. The distribution of the formate activating enzyme and other enzymes involving tetrahydrofolate in animal tissue. *Comp. Biochem. Physiol.* 1: 227-247.

WHITELEY, H.R., OSBORN, M.J. and HUENNEKENS, F.M. 1959. Purification and properties of the formate-activating enzyme from *Micrococcus aerogenes*. *J. Biol. Chem.* 234: 1538-1543.

WIELAND, O.P., HUTCHINGS, B.L. and WILLIAMS J.H. 1952. Studies on the natural occurrence of folic acid and *Citrovorum* factor. *Arch. Biochem. Biophys.* 40: 205-217.

WILLS, L. 1931. Treatment of "Pernicious Anaemia of Pregnancy" and "Tropical Anaemia". *British Med. J.* 1: 1059-1064.

WINSTEN, W.A. and EIGEN, E. 1950. Bioautographic studies with use of *Leuconostoc citrovorum* 8081. *J. Biol. Chem.* 184: 155-161.

WITTENBERG, J.B., NORONHA, J.M. and SILVERMAN, M. 1962. Folic acid derivatives in the gas gland of *Physalia physalis* L. *Biochem. J.* 85: 9-15.

WOODS, D.D., FOSTER, M.A. and GUEST, J.R. 1965. Cobalamin-dependent and independent methyl transfer in methionine biosynthesis. In: *Transmethylation and Methionine Biosynthesis*. (ed.) Shapiro, S.K. and

Schlenk, F., University Press, Chicago. p. 138.

WRIGHT, B.E. 1955. Polyglutamyl pteridine coenzymes. *J. Am. Chem. Soc.* 77: 3930-3932.

WRIGHT, B.E. 1955. A new cofactor in the conversion of serine to glycine. *Biochim. Biophys. Acta* 16: 165-166.

WRIGHT, B.E. 1956. The role of polyglutamyl pteridine coenzymes in serine metabolism. II. A comparison of various pteridine derivatives. *J. Biol. Chem.* 219: 873-883.

YEH, Y.-C. and GREENBERG, D.M. 1965. Purification and properties of $N^5$,$N^{10}$-methylenetetrahydrofolate dehydrogenase of calf thymus. *Biochim. Biophys. Acta* 105: 279-291.

ZAKRZEWSKI, S.F., HAKALA, M.T. and NICHOL, C.A. 1966. Purification and properties of folate reductase, the cellular receptor of amethopterin. *Mol. Pharmacol.* 2: 423-431.

B29995